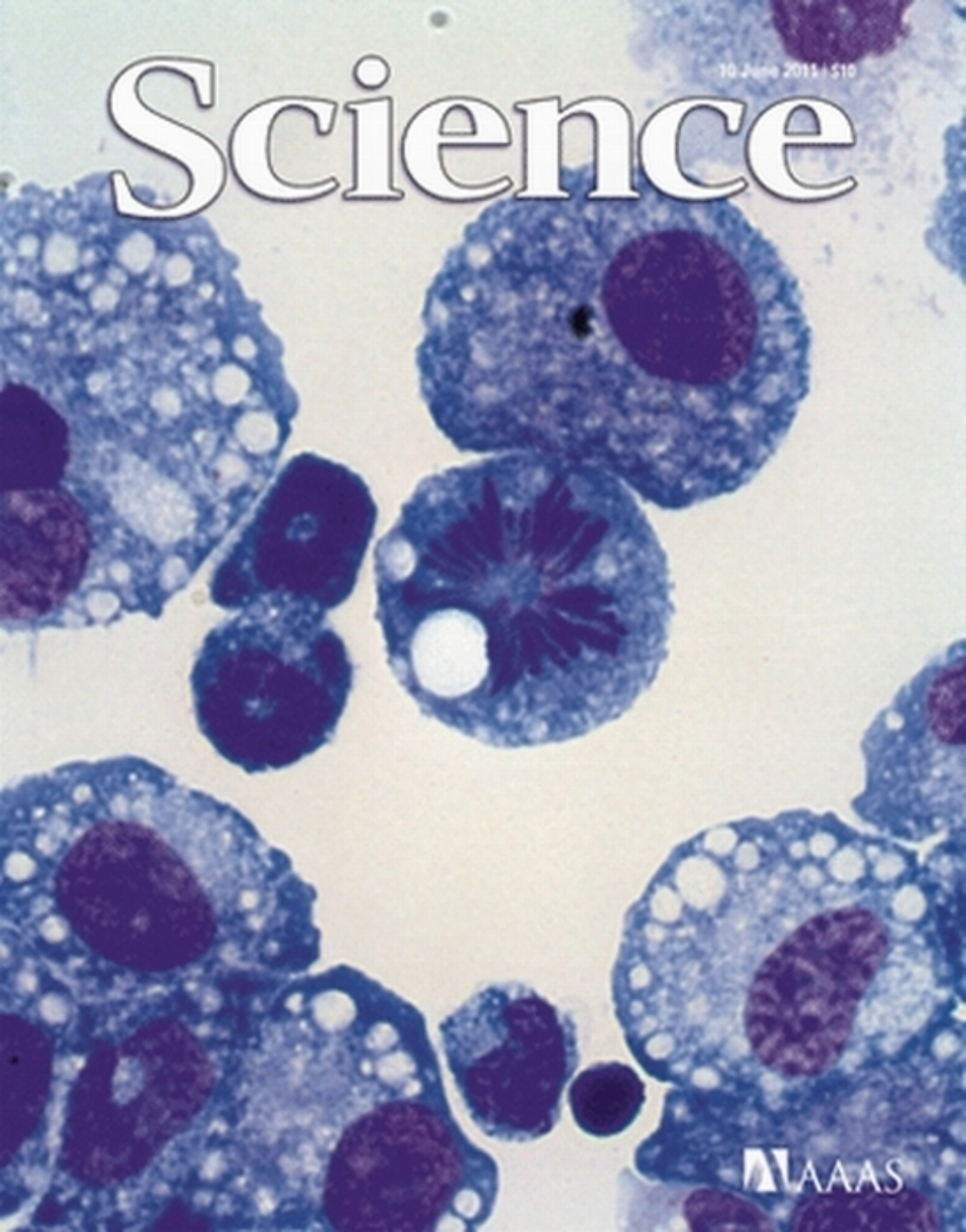




10 June 2015 | \$10

# Science

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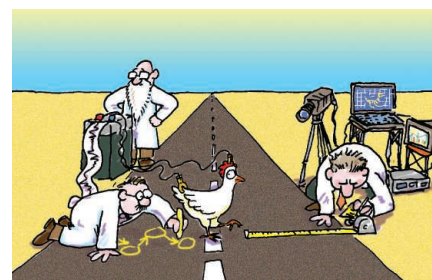
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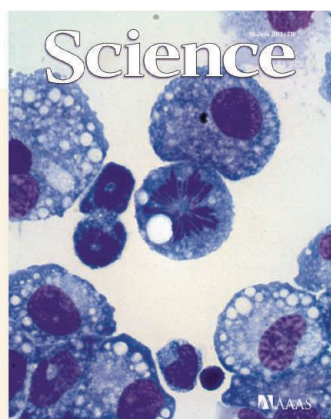
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## COVER

Bright-field light microscopy image of peritoneal cells, including a macrophage undergoing cell division (center cell, ~20 micrometers across), taken from mice treated with the cytokine interleukin-4. On page 1284, Jenkins *et al.* describe a mechanism of inflammation in response to parasitic nematode infection whereby macrophages expand in number through cell division rather than by recruitment from the blood.

Photo: Stephen Jenkins, University of Edinburgh, UK

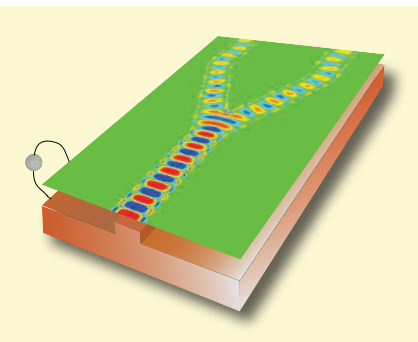
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## SCIENCEONLINE

## SCIENCEEXPRESS

[www.sciencexpres.org](http://www.sciencexpres.org)

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G. T. Pederson et al.

The snowpack covering the mountains of western North America has decreased dramatically during the past 50 years.

10.1126/science.1201570

### School-Based Early Childhood Education and Age-28 Well-Being: Effects by Timing, Dosage, and Subgroups

A. J. Reynolds et al.

A little bit of preschool goes a long way.

10.1126/science.1203618

>> [Science Podcast](#)

### Long Unfolded Linkers Facilitate Membrane Protein Import Through the Nuclear Pore Complex

A. C. Meinema et al.

Natively unfolded linkers facilitate nuclear membrane protein import.

10.1126/science.1205741

### Climate-Forced Variability of Ocean Hypoxia

C. Deutsch et al.

The spatial extent of ocean hypoxic zones, which are uninhabitable by many marine organisms, is very sensitive to dioxygen content.

10.1126/science.1202422

### Palladium-Catalyzed Aerobic Dehydrogenation of Substituted Cyclohexanones to Phenols

Y. Izawa et al.

Phenol derivatives are prepared from nonaromatic ring compounds that can bear a wide variety of substituents.

10.1126/science.1204183

## SCIENCENOW

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Highlights From Our Daily News Coverage

### Bat Hibernation Keeps Rabies Going

A mathematical model helps researchers understand how the virus spreads.

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### Antiatoms, All Out of Energy and Ready for Work

Reaching the least-energetic state is a milestone in efforts to compare antimatter and matter.

<http://scim.ag/antiatoms>

### Melanoma Drug Combo Shows Promise in Early Trial

A widely hailed new drug for melanoma, which was tested in combination with another drug to boost its tumor-fighting power, has done well in a key safety trial.

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## SCIENCE SIGNALING

[www.sciencesignaling.org](http://www.sciencesignaling.org)

The Signal Transduction Knowledge Environment

7 June issue: <http://scim.ag/ss060711>

### RESEARCH ARTICLE: TWIK-1 Two-Pore Domain Potassium Channels Change Ion Selectivity and Conduct Inward Leak Sodium Currents in Hypokalemia

L. Ma et al.

Very low extracellular K<sup>+</sup> concentrations, which can trigger cardiac arrhythmias, cause TWIK-1 potassium channels to become permeable to Na<sup>+</sup>.

### RESEARCH ARTICLE: The cis-Regulatory Logic of Hedgehog Gradient Responses—Key Roles for Gli Binding Affinity, Competition, and Cooperativity

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### PERSPECTIVE: Beyond the Balance of Activator and Repressor

T. Whittington et al.

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### RESEARCH ARTICLE: Antigen Potency and Maximal Efficacy Reveal a Mechanism of Efficient T Cell Activation

O. Dushek et al.

Efficient T cell activation depends on the rate with which T cell receptors and antigens bind and unbind, rather than simply their equilibrium affinity.

## GLOSSARY

Find out what BL, DUB, and FX mean in the world of cell signaling.

## SCIENCE TRANSLATIONAL MEDICINE

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Integrating Medicine and Science

8 June issue: <http://scim.ag/stm060811>

### PERSPECTIVE: Broken Barriers—A New Take on Sepsis Pathogenesis

N. M. Goldenberg et al.

Loss of the endothelial barrier and subsequent leakiness in the microvasculature might be key determinants of the pathogenesis of sepsis.

### RESEARCH ARTICLE: Protein Interactome Reveals Converging Molecular Pathways Among Autism Disorders

Y. Sakai et al.

An autism protein interaction network provides a framework for studying autism pathogenesis.

### RESEARCH ARTICLE: Off-Target Lapatinib Activity Sensitizes Colon Cancer Cells Through TRAIL Death Receptor Up-Regulation

N. G. Dolloff et al.

The breast cancer drug lapatinib increases the sensitivity of colon cancer cells to apoptosis through a HER2- and EGFR-independent mechanism.

### RESEARCH ARTICLE: Host Alloreactive Memory T Cells Influence Tolerance to Kidney Allografts in Nonhuman Primates

O. Nadazdin et al.

### PERSPECTIVE: Transplantation Tolerance—Memories That Haunt Us

M. L. Ford and C. P. Larsen

Screening for preformed donor-reactive memory T cells may advance the goal of organ transplant acceptance without immunosuppression.

## SCIENCE CAREERS

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I. S. Levine

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S. A. Holgate

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Science Policy News and Analysis

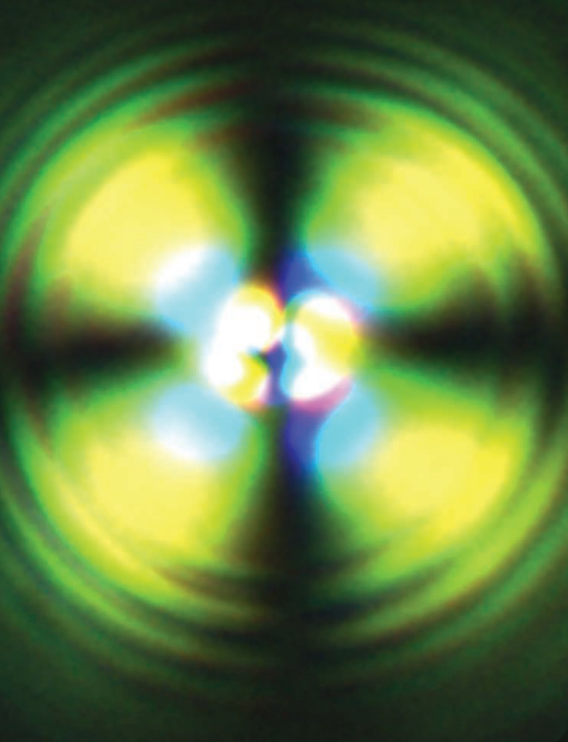
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## <<Endotoxin Revealed

Nematic liquid crystals are composed of rigid rod molecules that try to orient in the same direction on a local scale. When confined in certain geometries, the molecular ordering cannot satisfy the boundary conditions of the system. Instead, regions of low order in the form of point defects will form, which are easily observed using optical microscopy. **I.-H. Lin *et al.*** (p. 1297, published online 19 May) show that lipid A will interact with the defects in a liquid crystal droplet, allowing for the extremely sensitive detection of this glycopospholipid, an essential component of a bacterially produced endotoxin.

## Understanding Change

As a result of human activities, ecological communities are losing functionally irreplaceable species and gaining functionally novel ones, but research into the two processes has developed largely independently. **Wardle *et al.*** (p. 1273) review these two topics, explore how the species interchange may transform ecosystems through altering aboveground and belowground processes, and search for generalizations and unifying principles needed to understand how the Earth system may respond to global change.

## Macrophages Expand in Place

Macrophages play an important role in pathogen clearance during infections. Macrophages increase in number in infected tissues through the recruitment of monocytes from the blood, which then differentiate into macrophages. Although this is the case for inflammatory macrophages participating in classical “type 1” responses, **Jenkins *et al.*** (p. 1284, published online 12 May; see the cover; see the Perspective by **Randolph**) report a different mechanism for macrophage accumulation in tissues during “type 2” responses, which often occur in response to parasitic infection. Tissue resident macrophages proliferated in situ in response to infection with a filarial nematode. Proliferating macrophages had an “alternatively activated” phenotype and required the type 2 cytokine interleukin-4 for their expansion. This mechanism of macrophage expansion may promote parasite sequestration and tissue repair in the absence of

the immune pathology typically associated with the recruitment of inflammatory cells.

## Microcredit in the Philippines

One of the aims of microcredit—the granting of small loans to groups or individuals with limited access to traditional formal lending sources—is to spur the start or growth of small-scale businesses, especially those run by women. **Karlan and Zinman** (p. 1278) developed a randomized methodology for assigning microloans to individuals in the Philippines. The availability of microloans increased the amount of borrowing that individuals undertook, but their businesses did not grow and could actually shrink. However, microcredit fostered ties between these individuals and their communities, making risk more manageable and increasing access to informal lending.

## Graphene Transformation and Integrated Circuits

Metamaterials and transformation optics allow for the design of circuits and devices with functionalities beyond those of natural materials. **Vakil and Engheta** (p. 1291) show numerically that the optoelectronic properties of graphene offer the possibility of realizing such designer circuitry in a two-dimensional landscape only a single atom thick. The high mobility of charge carriers in graphene makes it an ideal candidate for the high-frequency devices used in radio-frequency applications. High-frequency, field-

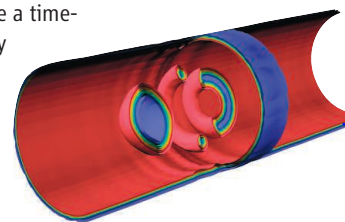
effect transistors have been demonstrated, but devices such as mixers, which create the sum and difference combination frequencies from two input frequencies, require other components.

**Y.-M. Lin *et al.*** (p. 1294) used electron-beam lithography to define the components of a mixer for graphene grown in silicon carbide.

## Superfluids in a Stir

Stirring a liquid can result in the formation of vortices or a change in the properties of the liquid, where a phase transition can be induced. If the liquid is a superfluid, quantum mechanical considerations restrict the angular momentum of the vortices so that they take quantized values and the liquid can become “normal.” Using somewhat phenomenological descriptions, how the vortices develop for a given condition is well understood, as are the different states that can arise, but a general theory describing the full evolution has been lacking. **Bulgac *et al.***

(p. 1288) describe a time-dependent density functional theory that provides a generalized description of the dynamics and phase transitions associated with the complex interactions of Fermi superfluids, which should prove a powerful tool in describing such systems.



## Through a Topsy-Turvy Tunnel

One of the counterintuitive consequences of quantum mechanics is that chemical reactions can sometimes proceed in systems that lack the energy needed to power the associated atomic rearrangements. This phenomenon is termed tunneling: The molecules pass through, instead of over, an energy barrier to products. **Schreiner *et al.*** (p. 1300; see the Perspective by **Carpenter**) describe a system in which tunneling completely upends the expected course of a molecular rearrangement. In an unstable carbene compound with OH and CH<sub>3</sub> groups bonded to a low-valency central carbon, an H atom shifted to the central carbon to form a more stable isomer. However, the H atom came from the OH group, despite facing a higher barrier than would a competing H shift from CH<sub>3</sub>.

*Continued on page 1239*

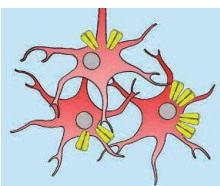
Continued from page 1237

## The Organic Who Fell to Earth

Carbonaceous chondrites are primitive meteorites that provide samples of the materials from which Earth and the other planets in the solar system formed. They contain organic matter, which, while it is thought to have formed in the solar protoplanetary disk or in the interstellar medium prior to the Sun's formation, could have suffered alteration inside the meteorite's parent asteroid. **Herd *et al.*** (p. 1304) studied the organic compounds present in four pieces of a well-preserved carbonaceous chondrite meteorite, the Tagish Lake meteorite. The data suggest that parent body hydrothermal alteration played a role in the preservation and creation of organic molecules.

## Seeing the Heat

Dim-light vision requires a visual system that has high sensitivity to light but a low probability of thermal activation. Nevertheless, thermal pigment noise does occur. **Luo *et al.*** (p. 1307) used single-cell recordings to measure photoisomerization activation energies and noise rates for diverse rod and cone pigments. A quantitative relationship was observed between a pigment's photoactivation energy and its peak absorption wavelength. A statistical-mechanical analysis using this relationship and modeling thermal activation was able to predict pigment noise rates.



## Smoking and Body Weight

Smokers are on average thinner than nonsmokers, and many smokers gain weight when they quit. However, the specific cellular mechanisms of nicotinic receptors responsible for the effects of nicotinic agents on feeding are unclear. Now, **Mineur *et al.*** (p. 1330) show that nicotine acts through  $\alpha_3\beta_4$ -containing nicotinic acetylcholine receptors to increase hypothalamic pro-opiomelanocortin neuron activity, which then decrease feeding and body weight. Thus, nicotinic agonists may be useful for limiting weight gain after smoking cessation, and nicotinic drugs could also help control obesity and related metabolic disorders.

## Mammalian DNA Damage Response

To identify previously unrecognized components of a mammalian DNA damage response system, **Cotta-Ramusino *et al.*** (p. 1313) used a large-scale screen of small interfering RNAs in a human tumor cell line. A group of about 100 genes were identified that were necessary to keep cells with damaged DNA from proceeding into mitosis. Two of the proteins that localized at sites of DNA damage were CLOCK and RHINO. CLOCK is a transcription factor that functions as part of the circadian clock, and RHINO is a component of a complex of proteins that act together to stimulate activity of the protein kinase ATR, which propagates the signal to arrest the cell cycle and allow DNA repair.

## mTOR Substrates Revealed

The protein kinase mTOR functions in protein complexes (mTORC1 and mTORC2) that are activated in response to agents such as growth factors and insulin and has important roles in regulating many physiological responses, including cell growth, proliferation, metabolism, and cell survival (see the Perspective by **Yea and Fruman**). **Hsu *et al.*** (p. 1317) and **Yu *et al.*** (p. 1322) conducted proteomic screens to identify substrates of mTORC1 and mTORC2 kinases. Numerous proteins that appear to be direct substrates were identified, including a new mTOR substrate, Grb10. Grb10 is an adaptor molecule that functions in the formation of signaling complexes associated with growth factor receptor tyrosine kinases.

## The Climes, They Are a-Changing

In the light of climate change, how will species adapt to changes in their current habitats and ranges? Using an ensemble of yeast populations across a gradient of salt to simulate different degrees of change across the environment, **Bell and Gonzalez** (p. 1327) explored the role of dispersal and evolution in explaining adaptation following environment deterioration in spatially structured populations. Adaptation was higher when there was local dispersal, and environmental degradation was gradual, and when previous adaptation to degradation had occurred relative to populations that had not previously experienced stressful conditions.

CREDIT: MINEUR ET AL.



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## Rekindling Japan's Spirit

AT THE END OF THIS MONTH, THE INTERNATIONAL BIO FORUM AND BIO EXPO JAPAN CONVENES in Tokyo, highlighting research, technologies, and biopharma trends from academic, industrial, and business sectors across Asia. For Japan, science is generally regarded as a means for boosting economic growth, and the nation has concentrated on supporting scientific discoveries for commercial applications. Basic science—which focuses on discoveries that explain how and why things work—has taken an unfortunate back seat. Yet, it is such curiosity-driven research that brings about conceptually novel discoveries and innovation. In the past decade, it has been difficult for young Japanese investigators in the life sciences to pursue this important type of science. Solutions to this critical problem may come by looking back in history, to a time when Japanese scientists knew how to appreciate nature and let that joy inspire their work.

The Edo period (1603 to 1868) was a time when the Japanese people faced neither domestic nor external battles. This peaceful environment allowed them to innovate in ways that fostered a scientific culture in concert with artistic activities. In the Ukiyo-e paintings by Jakuchu Ito and Hiroshige Utagawa, nature and living creatures were depicted with an amazing precision. Simultaneously, Edo biological and medical sciences developed within a creative framework that spurred discovery. Thus, for example, Seishu Hanaoka developed, for the first time, a herbal concoction as a general anesthetic.

Today, young Japanese researchers in the life sciences face a disheartening academic environment. Many broaden their knowledge and experience by doing postdoctoral work in leading laboratories in other countries, bringing new skills, ways of thinking, and great passion back to Japan as they begin to build their own laboratories in our universities. But ever-increasing administrative duties, incessant meetings, paperwork, and the constant writing of grant applications prevent them from using their time to think creatively—or even joyfully—about research. A decrease in the number of academic faculty positions in national universities has produced a highly competitive environment for young faculty members and a race to demonstrate success through maximizing publications, generating an environment in which Japanese researchers can rarely afford to enjoy the inherent beauty and wonder of the natural systems they are curious about.

Japanese scientists have made many unique contributions in the field of developmental biology, and here new model systems are emerging that have the potential to reignite the spirit that characterized the Edo period. For example, numerous strains of medaka—a popular fish in Japanese children's songs—maintained in Nagoya University piqued the curiosity of Hiroyuki Takeda and his colleagues (University of Tokyo), leading them to develop medaka as a fantastic animal model for studying genetics and development.\* Shigeru Kondo (Osaka University), who was curious about the striped skin of his personal pet, the angelfish *Pomacanthus imperator*, used it to develop a mathematical understanding of pigment pattern development.† And the famous Japanese morning glory asagao has been exploited by Eiji Nitasaka (Kyushu University) to analyze genetic transposition mechanisms.‡ In such ways, we are learning how diverse creatures evolved during animal and plant evolution. And because all organisms use the same molecular machinery, insightful, creative studies of unique organisms such as these can be powerful shortcuts for understanding the fundamental mechanisms that appear throughout nature, including in humans.

There is a popular old Japanese saying, “Onko-chishin,” or “Learn a lesson from the past.” The ignorance of history, coupled with an environment that discourages creative scientific thinking and wonderment, is a poor combination for both Japan and the Asian biotechnology industry, as the upcoming meeting in Tokyo should bear in mind.

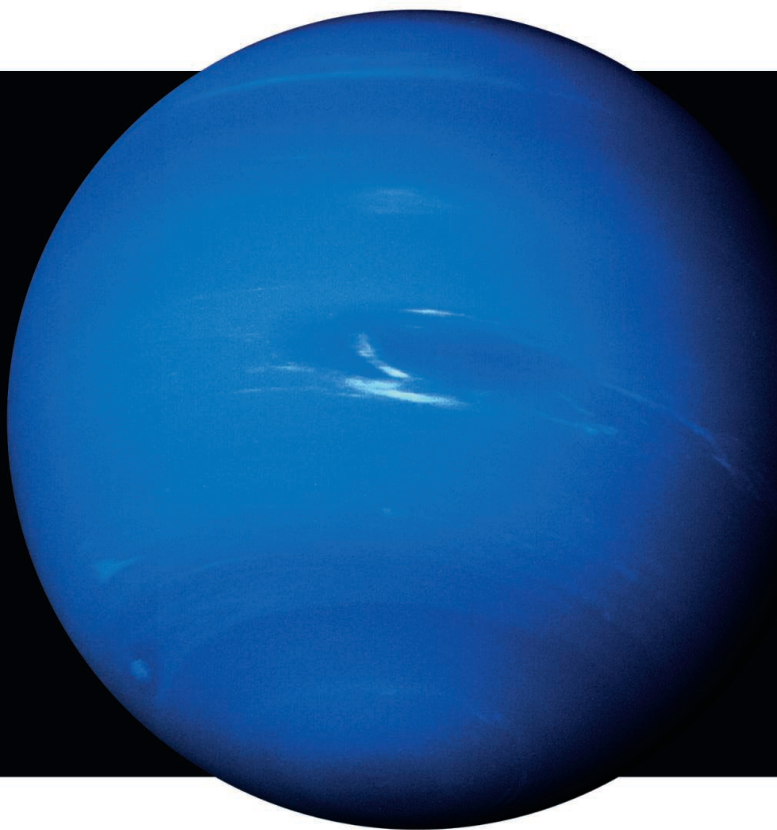
– Yoshiko Takahashi

10.1126/science.1209050



\*H.Takeda, A. Shimada, *Annu. Rev. Genet.* **44**, 217 (2010). †S. Kondo, T. Miura, *Science* **329**, 1616 (2010). ‡S. Kawasaki, E. Nitasaka, *Plant Cell Physiol.* **45**, 933 (2004).





## ASTROPHYSICS

### Placing Uranus and Neptune

The solar system has two reservoirs of comets: the Kuiper Belt, where Pluto is also located, and the Oort cloud, much further beyond the orbits of the planets. The comets did not necessarily form in the regions they now occupy. In the case of the Oort cloud, computer simulations of its formation suggest that it originated from the region where Uranus and Neptune once were. Based on this constraint and the similarity between the deuterium enrichment in the ice of Oort cloud comets and in Saturn's moon Enceladus, Kavelaars *et al.* suggest that at the time the Oort cloud formed, Uranus and Neptune were much closer to Saturn than they now are. Deuterium enrichment varies with distance to the Sun and is linked to the time and location at which these ices condensed. The result is consistent with models of the dynamical evolution of the solar system wherein Uranus and Neptune migrate from their formation region to their current location via dynamical interactions. — MJC

*Astrophys. J.* **734**, L30 (2011).

## IMMUNOLOGY

### Natural Helper Cells Hinder, Too

Viral infections in people with asthma can be particularly bad, because these patients are more prone to develop airway hyperreactivity (AHR) and inflammation, which can lead to substantial morbidity and even death. AHR is also seen in allergic asthma, but conventional therapies used for this disease are often not effective against virus-induced AHR, which suggests different underlying mechanisms of disease development and pathology. Chang *et al.* sought to determine the cellular and molecular mechanisms that drive AHR in response to viral infection by using mice infected with influenza virus. Infected mice rapidly developed AHR and inflammation that, instead of being dependent on the classical T helper type 2 cytokines typically associated with allergic asthma and AHR, were dependent on interleukin-33 (IL-33). Also unlike allergic asthma and AHR, influenza-induced AHR did not depend on the adaptive immune response. This led the authors to test the role of natural helper cells, an IL-33-secreting, newly described immune cell type that promotes immunity to intestinal helminth infections. Indeed, depletion of natural helper cells blocked the development of AHR in influenza-infected mice, and transfer of these cells to IL-33-deficient, influenza-infected mice

restored AHR. Although these results await confirmation in humans, they suggest that, like other immune cell subsets, natural helper cells can have both protective and pathological effects. — KLM

*Nat. Immunol.* **12**, 10.1038/ni.2045 (2011).

## CELL SIGNALING

### Taking Sleep Up a Notch

Notch receptor signaling is best known for its regulation of pattern formation and cell fate during development, but recent evidence also indicates an important role for Notch in the adult brain. Two new studies now show that Notch signaling regulates sleep or sleep-like functions in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. In *Drosophila*, Seugnet *et al.* found that a negative regulator of Notch signaling accumulated after sleep deprivation.

Restoration of Notch signaling decreased the amount of extra sleep required by sleep-deprived flies and prevented the deficit in learning caused by loss of sleep. In *C. elegans*, Singh *et al.* showed that Notch signaling promoted a sleep-like quiescent behavior normally associated with larval molting. Mutations in various Notch signaling components that led to a reduction of Notch signaling also lowered the arousal threshold from this sleep-like state, thus affecting the quality of sleep. Although the specifics of how Notch signaling affects sleep may vary between different organisms, these studies hint that reduced Notch signaling is associated with responses to environmental stress. — LBR

*Curr. Biol.* **21**, 835 (2011);

*Curr. Biol.* **21**, 825 (2011).

## PSYCHOLOGY

### Shame and Honor Work

Humans have evolved both rewards and punishments to dictate those behaviors that are acceptable and those that are not. The extent to which shame and honor affect individual decisions in group situations where cooperation leads to reduced individual benefit, however, is not well understood. Jacquet *et al.* used anonymous donations as a means to separate out behavior stimulated by honor and shame by

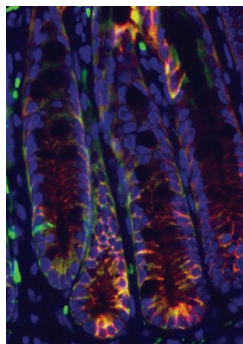


exposing those who had given the most versus the least in a public goods game setting. Both honoring those who donated the most as well as shaming those who donated the least led to an approximate 50% increase in cooperation, showing how the desire to avoid shame and gain honor shapes an individual's behavior. — LMZ  
*Biol. Lett.* **7**, 10.1098/rsbl.2011.0367 (2011).

## BIOMEDICINE

## Deconstructing a Probiotic

Despite aggressive marketing campaigns that highlight the beneficial effects of probiotics (therapeutics consisting of live microorganisms) on gastrointestinal health, in many cases the claimed benefits are made on the basis of



limited or controversial clinical data. Moreover, there are far more hypotheses than experimental data on the molecular mechanisms by which probiotics alter gut homeostasis. A better understanding of these mechanisms could shed light on the disparate clinical data and perhaps even lead to more effective drugs that can substitute for living microbes.

Studying *Lactobacillus rhamnosus* GG (LGG), which is used in yogurt as a nutritional supplement, Yan *et al.* found that this bacterium secretes a soluble protein, called p40, which prevents death of intestinal epithelial cells through activation of the epidermal growth factor receptor signaling pathway. In three mouse models of intestinal inflammation, administration of recombinant p40 (encased within special hydrogel beads to minimize its degradation) reduced disease symptoms in both a therapeutic and preventive setting. Whether p40 alone will show similar activity in humans without adverse side effects remains to be seen, but these results support the idea that the identification of the specific health-promoting factors made by probiotics is an avenue worth exploring. — PAK

*J. Clin. Invest.* **121**, 10.1172/JCI44031 (2011).

## CHEMISTRY

## A Fiery By-Product

Fire has alternately salvaged and savaged human beings since the dawn of civilization. It's rather remarkable, after so much time, that the combustion process still holds mysteries for chemists to probe. One such mystery is unraveling the

extraordinarily complex composition of the smoke-borne by-products that accompany release of the primary products, water and carbon dioxide. Among these by-products are a range of highly reactive small molecules that may cause physiological damage upon inhalation. Roberts *et al.* chart the abundance of one such molecule, isocyanic acid, by systematically probing the product stream of controlled laboratory biomass fires using a mass spectrometric technique especially sensitive to acids (negative-ion proton-transfer chemical ionization). In addition to documenting concentrations from these laboratory experiments of 600 parts per billion by volume, the authors detect hundreds of parts per trillion of the compound in urban Los Angeles air and in Boulder, Colorado, air after a wildfire. They furthermore estimate significant aqueous solubility of the compound at physiological pH, based on a Henry's Law partitioning measurement. Because previous studies have implicated isocyanic acid and its conjugate base in inflammation effects associated with protein carbamylation, the authors urge further study of the compound's exposure impact. — JSY

*Proc. Natl. Acad. Sci. U.S.A.* **108**, 8966 (2011).

## PHYSICS

## Quantum Conferencing

For certain critical transactions and communications, you want to be secure in the knowledge that your message cannot be stolen or compromised by a malicious hacker. Encrypting messages with keys distributed beforehand to all interested parties is the usual method to ensure security. For ultimate or unconditional security, however, one key per message or transaction is allowed, after which the key is discarded. This "one time pad" requirement can place a hefty overhead on distributing the keys and would not be particularly practical for everyday use. In quantum key distribution (QKD), the encryption keys are made up of a series of quantum bits, single photons of light, for instance, with the orthogonal polarization states encoding a logical 1 or 0. Because the bits are quantum mechanical in nature, any attempt by an eavesdropper to measure them would compromise that effort by a telltale sign. Sasaki *et al.* have now demonstrated the feasibility of quantum key distribution over an optical network in and around the metropolitan Tokyo area. Meshing together six separate QKD systems, they achieve secure video conferencing, encrypted with quantum keys, over a distance of 45 km. Stable operation and interfacing to the mobile telephone network widens the possible applications of quantum security. — ISO

*Opt. Express* **19**, 10387 (2011).

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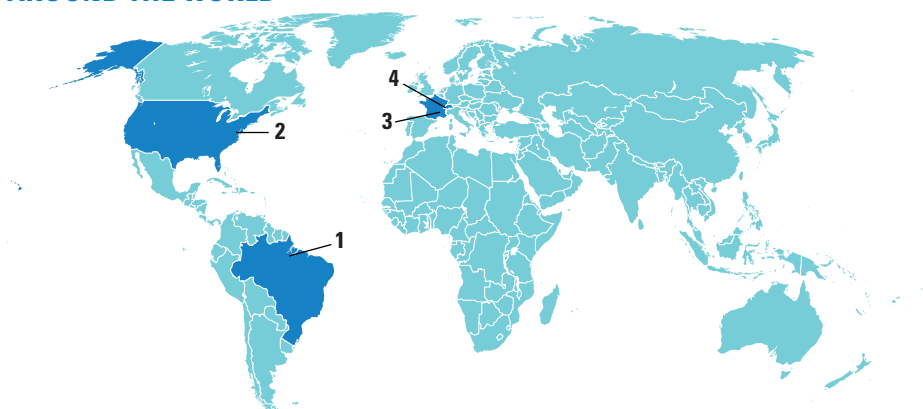
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**'Monster Dam' Gets Green Light**

Brazil's environment agency gave final approval last week for the construction of the Belo Monte Dam, an immense hydroelectric station on the Xingu River in the Amazon rainforest.

The dam, in planning for 30 years, is one of several large projects being built on tributaries of the Amazon River that are expected to damage pristine regions. Earlier this year, 20 Brazilian scientific societies wrote to Brazilian President Dilma Rousseff opposing the dam, saying it would violate the human rights of local indigenous groups.



The new dam would straddle the Xingu River in the Amazon.

But on 1 June, Brazil's environmental agency, Ibama, issued a license to the consortium Norte Energia SA to begin construction. The \$16 billion dam would flood 516 square kilometers of forest while lowering water levels in the Xingu River, which locals say they depend upon for fishing.

Designers say the 6-km-wide dam, slated for completion in 2015, would produce more than 11,233 MW of power, ranking it as the third largest in the world and helping Brazil stay in the top tier of renewable energy users. An analysis by antidam campaigners predicts 40,000 people could be displaced by the dam; the Brazilian government's estimate was 20,000. <http://scim.ag/belo-monte>

Washington, D.C. 2

**Homeland Security Science Under Attack**

Science and technology programs at the U.S. Department of Homeland Security (DHS) would be drastically downsized under a spending bill passed last week by the House of Representatives. The legislation would reduce funding for the science and technology directorate by 52%, from this year's \$827 million to \$398 million in fiscal year 2012, which begins on 1 October. By comparison, the Obama Administration requested \$1.2 billion for those programs in 2012. The cuts are part of a \$1.1 billion reduction in the agency's overall budget, now \$43.4 billion, and are in line with a plan by House Republicans to rein in spending across the government.

DHS officials say the cuts will wipe out dozens of programs, stalling the development of technologies for border protection, detection of biohazards, and cargo screening. Agency officials hope the Senate will restore some of the funds when it takes up the spending bill in the coming weeks. But a final number is unlikely until the White House and Congress agree on government-wide spending levels and raising the debt ceiling, contentious issues that may take months to resolve.

Lyon, France 3

**WHO Weighs In on Cell Phones and Cancer**

Whether cell phones cause brain cancer has been debated for years, and last week the World Health Organization (WHO) stepped into the fray. A committee of 31 experts that had spent 8 days in Lyon, France, reviewing the literature concluded



that radio-frequency electromagnetic fields, including those from cell phones, are "possibly carcinogenic" to people.

The International Agency for Research on Cancer's (IARC's) uncertain classification already applies to more than 250 other agents, including lead and engine exhaust. In fact, it's so tough to show that something doesn't cause cancer that only one of the 900 or so agents that IARC has evaluated falls in the "probably not carcinogenic" category.

The working group was swayed partly by a study called Interphone. It reported last year that although cell phones didn't seem to be linked to brain cancer in most people, for heavy cell users there were hints of risk.

Reaching a more definitive answer won't be easy; one reason is that epidemiologic studies like Interphone depend on people recalling their cell phone use. A European effort called COSMOS is recruiting about 250,000 people and will track their cell phone use with help from phone company records. But it's years away from reporting results. <http://scim.ag/WHO-phones>

Geneva, Switzerland 4

**AIDS Turns 30**

Thirty years after the epidemic first surfaced, just over 34 million people live with HIV. But by the end of last year, antiretroviral (ARV) drugs reached more people in resource-constrained countries—6.6 million—than ever before, says a new report from the Joint United Nations Programme on HIV/AIDS (UNAIDS), released 2 June. Infection rates, too, plummeted 25% worldwide between 2001 and 2009. But the report, titled *AIDS at 30*, warns that international funding needed

**NOTED**

>Good news: You can now download [that obscure National Academies report](http://www.nap.edu/) you wanted for free. The National Academies Press (<http://www.nap.edu/>) announced on 2 June that it was extending the new low price, currently available on 65% of its PDFs, to the rest of more than 4000 digitized books, as well as future reports.

to maintain progress has been declining.

Even though the world now spends \$15.9 billion a year combating the epidemic, 9 million people who need treatment still have no access to ARVs, says the report. And more than two dozen countries offer ARVs to just 20% or fewer of their citizens who need treatment. UNAIDS estimates that increasing the number of people being treated to 15 million by 2015 will cost \$22 billion a year.

The report ushers in this week's U.N. General Assembly High-Level Meeting on AIDS, which will hammer out a new declaration about how best to address the epidemic. Michel Sidibé, executive director of UNAIDS, says that developing countries will be asked to shoulder more of the burden. <http://scim.ag/AIDS-30>

## NEWSMAKERS

### Trailblazing Nobelist Passes

"She is from New York. She is Jewish. She is a woman."

That's what a prominent U.S. university wrote about Rosalyn Yalow while denying her a spot in its graduate program decades ago. Yalow, who went on to win a Nobel Prize in physiology or medicine, died on 30 May in New York City. She was 89.

Born to parents who never went to college, Yalow decided at age 8 to become a scientist. She became the first woman to earn a bachelor's degree in physics from NYC's Hunter College. But no graduate program accepted her until World War II, when the University of Illinois offered her a teach-



ing assistantship. She got her doctorate in nuclear physics from Illinois in 1945.

She began working at the Bronx Veterans Administration Hospital in 1950. There she and Solomon Berson developed the radio-immunoassay for measuring minute quantities of hormones in blood with radioactive tracers. The technique, which can also detect drugs and viruses, ushered in a "revolution in biological and medical research," the Karolinska Institute in Sweden said when Yalow was awarded the Nobel Prize in 1977.

### Tracers of the Universe's Architecture

In the early 1980s, scientists thought that dark matter, the mysterious stuff whose gravity binds galaxies, might actually consist of familiar, nearly massless particles called neutrinos. Then cosmologists Marc Davis of the University of California, Berkeley; George Efstathiou, now at the University of Cambridge in the United Kingdom; Carlos Frenk, now at Durham University in the United Kingdom; and Simon White, now at the Max Planck Institute for Astrophysics in Garching, Germany, proved that convenient idea untenable. Instead of "hot" neutrinos zipping about at near-light speed, dark matter had to consist of beefy, slow-moving, "cold" particles unlike any yet observed. That insight and the methods used to achieve it have won the quartet the 2011 Gruber Cosmology Prize and \$500,000 to share.

Using large-scale simulations, pioneered by Efstathiou, they showed that only cold dark matter would lead to the distribution of galaxies observed in so-called redshift surveys, pioneered by Davis. "Nobody

took it [cold dark matter] seriously until the four of them did their first large-scale simulations," says Julio Navarro, a cosmologist at the University of Victoria in Canada and a member of the prize committee. He adds: "They really started a whole industry in using this kind of simulation, ... which has become a primary tool in cosmology."



Clockwise from top left: Davis, Efstathiou, White, and Frenk.

### Shaw Prizes

The 2011 Shaw Prizes, announced this week, highlight achievements in astrophysics, immunology, and geometry. Enrico Costa of the Institute of Space Astrophysics and Cosmic Physics in Rome and Gerald Fishman of NASA's Marshall Space Flight Center in Huntsville, Alabama, share the astronomy prize for leading the development of space instruments that determined that gamma ray bursts—fleeting, energetic flashes of gamma rays—result from violent explosions in galaxies billions of light years from Earth. Discovering the molecular mechanism of innate immunity, which is the first line of defense against pathogens in all plants and animals, nets the life science and medicine prize for Jules Hoffmann of the University of Strasbourg in France, Ruslan Medzhitov of Yale University, and Bruce Beutler of the Scripps Research Institute in San Diego, California. And Demetrios Christodoulou of ETH Zurich and Richard Hamilton >>

### Time Capsule From the Atomic Age

On 2 June, a crowd watched as workers pried a large slab from the corner of the Research Institutes building at the University of Chicago (U of C) to reveal a small box, placed there in 1949 by the legendary physicist Enrico Fermi, pioneer of the nuclear reactor. The crowd waited, hushed. What had Fermi hidden away for the generations? Scientific secrets? Predictions for the future?

Instead, master of ceremonies Roger Hildebrand, a U of C physicist and younger contemporary of Fermi's, held up a slim orange volume. "This is the telephone directory from the University of Chicago in 1948," he announced. Next came a road map from a gas station, followed by a bundle of airline timetables (including a five-stop one-way flight to San Francisco for \$113.75). And that was pretty much that.

In a TV interview after the event, U of C physicist Riccardo Levi-Setti, who first met Fermi as a Ph.D. student in 1949, seemed stunned: "I'm a little surprised that there wasn't really"—he paused for a long time—"something very inspiring." The building will be demolished in August to make way for the new Eckhardt Research Center.





## Random Sample

## Mating of the Titans

**BEIJING**—As the scalpel sliced into the towering celebrity, a crowd gathered to watch history in the making. A few cuts later, just before 9:00 p.m. on 28 May, Niu Xia removed a 5-centimeter-square chunk of green flesh and inserted a syringe. The operation was a success, but still the patient had only hours to live.

The celebrity was titan arum, or corpse flower (*Amorphophallus titanum*), which boasts the tallest unbranched inflorescence on the planet, sometimes topping 3 meters. Famously hard to cultivate, the plant offers a noxious reward: When its flower-like spathe opens, the tiny female flowers inside stink like a rotting corpse. That attracts its pollinators: carrion-eating beetles and flesh flies.

In one respect, the corpse flower truly is the living dead. Cultivated individuals almost always begin dying hours after blooming. Sure enough, the night after the operation at Beijing Botanical Garden (BBG), the titan's spadix collapsed. That devastated Niu, who had raised the plant from when it was a mere tuber. "She cried all night," says fellow BBG horticulturalist Guo Ling. "It was like it was her baby."

The withering titan arum may yet have babies of its own. Half a world away, scientists at the Royal Botanic Garden, Edinburgh, in the United Kingdom will attempt to pollinate their own first-time bloomer with the Chinese plant's pollen. As Niu sliced into the spathe and extracted the yellow sacs, BBG Director Zhao Shiwei hovered like an expectant father. In a few weeks, Zhao will learn whether the Scottish plant has borne a cluster of 500 or so dark-orange berries. Then he can break out the cigars.



## BY THE NUMBERS

**1,211,287** Square kilometers of ice road-accessible Arctic lands that will be unreachable by 2050, a 14% decrease, according to a report online 29 May in *Nature Climate Change*. Iceland alone will lose 82%.

**8** Points out of 72 that a U.K. university admissions exam devoted to a math problem later found to be unsolvable. Nearly 6800 students took the test on 26 May.

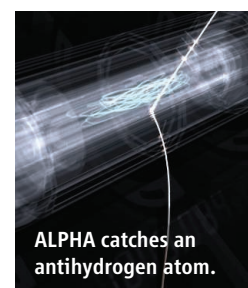
## Antiatoms, Ready for Work

Six months ago, physicists trapped atoms made of antimatter for a fraction of a second. Now, the same team, which works with the ALPHA experiment at the European particle physics lab, CERN, near Geneva, Switzerland, has held individual atoms of antihydrogen, each consisting of an antiproton bound to a positron, for up to 15 minutes. That's long enough for an atom to radiate all of its internal energy and settle into its "ground state," a prerequisite for probing its inner workings.

The result, reported online 5 June in *Nature Physics*, brings physicists closer to their decades-old goal of precisely comparing the spectrum of light absorbed by antihydrogen to that of hydrogen. Any difference in the spectra would spoil a key symmetry thought to exist between matter and

antimatter and, hence, a symmetry of space and time that undergirds Einstein's theory of special relativity.

Measuring antihydrogen's spectrum with sufficient precision may take years, however, and ALPHA



ALPHA catches an antihydrogen atom.

faces competition from two other experiments, also at CERN. "This is clearly a very, very important experimental step forward," says University of Tokyo physicist Ryugo Hayano, who leads the rival ASACUSA team. "But that doesn't mean the next step is going to be easy."

<http://scim.ag/anti-H>

## &gt;&gt;NEWSMAKERS

of Columbia University take the mathematics prize for their work on nonlinear partial differential equations in Lorentzian and Riemannian geometry and their applications to general relativity and topology.

Hong Kong media mogul and philanthropist Run Run Shaw established the prizes in 2002. The winners in each category will split \$1 million to be awarded at a ceremony in Hong Kong in September.

## FINDINGS

## New Superbug Found in Cows and People

A novel form of deadly drug-resistant bacteria has turned up in dairy cows and humans in the United Kingdom. But don't toss that milk just yet: The superbug isn't a concern in pasteurized dairy products.

The bug is methicillin-resistant *Staphylococcus aureus* (MRSA), a drug-resistant form of the normally harmless *S. aureus* bacterium. It preys on people with weakened immune systems, causing about 19,000 hos-

pital deaths a year in the United States.

Mark Holmes of the University of Cambridge in the United Kingdom and colleagues stumbled upon a new MRSA strain while studying mastitis, or infected udders, in U.K. dairy cows. While clearly resistant to antibiotics, the strain came back negative on a standard PCR test for *mecA*, a gene found in all MRSA strains. Only by sequencing the bacterium's entire genome did the team find the gene in an altered form.

About two-thirds of 74 human samples of drug-resistant *S. aureus* from the United Kingdom and Denmark, despite also failing the usual PCR test, carried the new *mecA*, the team reported online 3 June in *The Lancet Infectious Diseases*. A nearly identical *mecA* gene has also been reported in human MRSA samples from Germany and Ireland.

The strain probably makes up less than 1% of all detected MRSA cases, the team says. And infected cows probably don't pass the bug to humans through milk, which is almost always pasteurized, but via contact with dairy workers who may become carriers. <http://scim.ag/new-MRSA>





**Dangerous.** A German researcher holds a culture of *E. coli* from a patient.

GERMANY

## Scientists Rush to Study Genome of Lethal *E. coli*

When cholera raged in the German port city of Hamburg in 1892 and killed thousands of people, famous epidemiologist Robert Koch pinpointed contaminated drinking water as the source of the infection, but he was unable to isolate the responsible bacterium. Nearly 120 years later, German public health officials and scientists are facing the opposite dilemma.

As *Science* went to press, they had not been able to find the source of the deadliest outbreak of enterohemorrhagic *Escherichia coli* (EHEC) bacteria on record. Yet they are getting to know the pathogen causing it in unprecedented detail, aided by an armada of scientists around the world who are analyzing available genomic data on the fly and, via tweets, wikis, and blogs, disseminating results online. "I am really surprised and impressed at how fast this is developing," says Holger Rohde, a microbiologist at the University Medical Center Hamburg-Eppendorf. "I think it shows how relevant this platform can be to science."

Although *E. coli* are a natural part of the human gut flora and usually not pathogenic, the strains classed together as EHEC produce

the dangerous Shiga toxin that enters the cells lining the gut and inhibits protein synthesis. The resulting cellular destruction leads to abdominal cramping and eventually bloody diarrhea. In some cases, the toxin also attacks the kidneys, triggering the potentially fatal hemolytic-uremic syndrome (HUS). During the outbreak that started the second week of May in northern Germany, more than 2300 people had become infected as of 7 June, more than 600 had developed HUS, and at least 23 had died.

As the number of EHEC cases started to rise in Germany, microbiologists at the University Medical Center Hamburg-Eppendorf, the clinic hit hardest by the outbreak, were swamped by patient samples to be examined. But then a Danish postdoc of Chinese origin working there on an exchange program raised the idea of teaming up with the Beijing Genomics Institute (BGI) in Shenzhen to sequence the genome of the deadly bacterium. On Wednesday 25 May, the clinic sent a small tube of purified bacterial DNA to BGI. "It arrived in China on Friday and the sequencing started on the weekend," Rohde says.

On 2 June, Chinese scientists announced

that they had deciphered the microbe's entire 5.2-million-base-pair genome and immediately made the DNA sequence available for researchers to download. Scores of scientists all over the world started poring over the data, assembling sequence fragments generated by BGI into a coherent genome, and comparing it to reference genomes for *E. coli* and other bacteria. The same day, a collaboration between the University of Münster and Life Technologies Corp., which manufactures advanced DNA sequencing machines, announced it had also sequenced a strain from a patient.

The two announcements came on the second day of a U.K. meeting on applied bioinformatics and public health microbiology. Speakers and other attendees immediately started working on annotating the bacterial sequence provided by BGI. "In less than 24 hours we got the reads, the assembly, and the annotation. A good case study," blogged Marina Manrique of era7 bioinformatics, a Spanish company that quickly did an automated analysis of the *E. coli*'s genome.

The picture emerging from these first analyses is surprising: The German strain's DNA sequence revealed the microbe not to be a typical EHEC bacterium. Instead, the pathogen shares 93% of its sequence with EAEC 55989, an *E. coli* strain isolated in 2002 from an HIV-positive patient in the Central African Republic suffering from chronic diarrhea. EAEC stands for enteroaggregative

*E. coli*, which can form biofilms and are better at colonizing the human gut than EHEC, says Lothar Beutin, head of the National Reference Laboratory for *E. coli* at the Federal Institute for Risk Assessment in Berlin. They usually do not carry the gene for the Shiga toxin, however. “The sequence really looks like a typical EAEC that has acquired the Shiga toxin gene,” Beutin says.

Other scientists agree based on the data so far. That’s the “most likely explanation,” says Frederick Blattner of the University of Wisconsin, Madison, who led the effort to sequence *E. coli*’s genome for the first time, a project that finished in 1997 and took more than a decade.

The combination of EAEC backbone and Shiga toxin might explain some of the unusual characteristics of the German outbreak: the high number of HUS cases, for example, and the fact that so many young, healthy adults are becoming sick. “At first, we thought that the severity of the cases might be due to some change in the toxin,” Rohde says. “But now it seems more likely that is just the fact that these bacteria form fimbriae.” Fimbriae are small tentacles that help EAEC bacteria stick to the intestinal wall. This could lead to a stronger colonization of the gut and more toxin being released into the body, Rohde and other scientists suggest.

Other questions remain, however. Why are women sickened more often? And what is the source of the infections? Early on in the outbreak, an epidemiological study conducted in Hamburg found that women who had become infected were more likely than uninfected women to have eaten raw tomatoes, cucumbers, and lettuce in the days before falling ill. German authorities then pointed to Spanish cucumbers as a likely source, but the *E. coli* strain isolated from them did not match the one isolated from patients. Sprouts from a grower that had delivered to a number of restaurants implicated in the outbreak were the next suspect, but, as *Science* went to press, the first samples from that source did not test positive for the bacteria either.

Early speculations that the bacteria were transmitted to vegetables through the use of manure are now challenged by the new genetic findings. While EHEC are found in the gastrointestinal tract of ruminants, EAEC have a reservoir in humans. “These bacteria are adapted to the human gut, and that is where you find them,” Beutin says.

It now looks likely that more than 10 years ago one of these bacteria acquired the Shiga toxin gene. Scientists at the National Consulting Laboratory on Hemolytic Uremic Syndrome in Münster had announced early on in



**Tossed salad.** European countries dumped Spanish cucumbers on false fears that they were infected.

the outbreak that, based on the PCR analysis of a few genes, the current strain matched an isolate taken from HUS patients in Germany in 2001. They have now sequenced that genome as well and have started to compare it with the isolates of the current outbreak. Preliminary data suggest that the 2001 strain is even more closely related to the current outbreak strain than is the one from Africa. Whereas the strain isolated in Africa was a typical EAEC, the bacterium in the 2001 German isolate had already acquired the gene for the Shiga toxin. The current strain has now acquired a host of new resistances to common

antibiotics. “You can almost see this bacterium changing,” Rohde says.

Some scientists are suggesting that a new class of pathogenic *E. coli* that is both enterohemorrhagic and enteroaggregative has been found. “We probably need a new name for such bacteria,” Beutin says. But an enteroaggregative *E. coli* producing the Shiga toxin has been reported once before. German, French, and Italian researchers together published a paper in 1998 describing such a strain as the cause of an outbreak of HUS cases. That team suggests the current German strain might not be the result of some rare event. “This is not a new germ arising from a strange recombination but an established pathogen that has acquired the Shiga toxin,” Stefano Morabito of the Italian Istituto Superiore di Sanità, one of the authors of the 1998 paper, says.

One lesson from this outbreak, Rohde says, “is that we just do not know enough about the flexibility of bacterial genomes, about how they constantly change.” Until we do, researchers warn, it is also hard to judge whether the German outbreak is an unlikely tragedy or something that could happen again elsewhere very soon.

—KAI KUPFERSCHMIDT

Kai Kupferschmidt is a writer in Berlin.

#### NEWSMAKER INTERVIEW: JOHN MASHEY

## Computer Scientist Goes on Offensive To Defend Climate Scientists

To climate scientists like Pennsylvania State University’s Michael Mann, who has come under relentless attacks from climate change skeptics, John Mashey is “one of the good guys.” The 65-year-old Mashey, who amassed a small fortune designing computer systems for the likes of Bell Labs and Silicon Graphics, is spending his retirement years compiling voluminous critiques of what he calls the “real conspiracy” to produce “climate antiscience.” He is trying to turn the tables, using tactics some of Mann’s opponents may find uncomfortably familiar.

Will Happer, a physicist at Princeton University who questions the consensus view on climate, thinks Mashey is a destructive force who uses “totalitarian tactics”—publishing damaging documents online, without peer review—to carry out personal vendettas. Whereas Mann lauds Mashey for “exploring the underbelly of climate denial,” Happer says Mashey’s

tactics are “contrary to open inquiry.”

Both sides can agree on one thing, however: Mashey has become one of the most visible of a new generation of citizen climate warriors. This month he scored a prominent victory when the journal *Computational Statistics & Data Analysis (CSDA)* retracted a 2008 paper co-authored by economist and climate skeptic Edward Wegman. Mashey had attacked the article last September in a 250-page analysis released online and headlined “Strange Scholarship in the Wegman Report.” Last week, the journal’s editors issued a statement saying that the article contained “portions of other authors’ writings on the same topic in other publications, without sufficient attribution to these earlier works being given.” To Mann, “the retraction validates [Mashey’s] efforts.”

Mashey’s critique also looked into a 2006 report to Congress by Wegman claiming that two paleoclimate studies by Mann and two



other authors used poor statistical analyses. Mashey blasted the science in the so-called Wegman report and alleged that many pages were copied from published sources without citation; no official action has been taken.

The *CSDA* paper grew out of a section of the Wegman report that asserted the authors' social connections had corrupted peer review of paleoclimate papers and that various independent studies "may not be as independent as they might appear." Wegman, a professor of statistics at George Mason University in Fairfax, Virginia, wrote in a March e-mail to the journal's publisher, Elsevier, which was investigating allegations of plagiarism, that he was "innocently unaware" that text on network theory and climate had been "copied and pasted" by a George Mason student into the Wegman report and later included in the *CSDA* paper. (*USA Today*, which first reported on the retraction, obtained the e-mail from *CSDA* and sent it for comment to Mashey, who published it.) Wegman and his lawyer declined several requests for comment from *Science*, but the latter told *USA Today* that neither Wegman nor the first author of the *CSDA* paper, an assistant professor at George Mason, "has ever engaged in plagiarism."

Mashey's career in computers only occasionally touched on climate issues, but his background has provided him with valuable skills for his new role. After earning a Ph.D. in computer science from Penn State in 1974, Mashey worked for several Silicon Valley companies designing systems for users that included intelligence agencies, oil companies, and climate scientists. He was

drawn into the swirling climate debate in 2007 after hearing a lecture on climate skeptics by University of California, San Diego, science historian Naomi Oreskes at Stanford University, which is near his Portola Valley home.

As he got to know Oreskes and allied climate scientists, Mashey became upset that they were being attacked by bloggers and lawmakers and subjected to anonymous threats. "Naomi's a friend, and she gets death threats. Mike Mann's a friend, and he gets death threats. It pisses me off," Mashey says. "They get harassed and discouraged for doing a good job for everybody's grandchildren." Defending their work from unfair attack is the least he can do, he reasons. "They seem heartened by the knowledge that somebody cares and is actually trying to take the offense."

Mashey decided to join the nascent community of nonscientist climate bloggers by preparing documents that dissected what the opponents were actually saying. (He maintains no blog but publishes documents through allied sites.) "Climate scientists do not have the skill set for investigating where weird stuff is coming from," Mashey says. He likens

**"Mike Mann's a friend, and he gets death threats. It pisses me off."**

—JOHN MASHEY

his current activities to the corporate intelligence efforts he once undertook and to investigations of organized crime. "You see what they're doing, who they talk to. Then what you do is, you hope they make a mistake."

His home office includes a laptop equipped with three extra screens to help him compile his reports. "When I'm trying to find these hidden connections or

Mashey drew Happer's ire with a 128-page report in 2009 critiquing a petition from Happer and other members of the American Physical Society urging the society to revise its call for cuts in greenhouse gas emissions and instead to question the existence of anthropogenic warming. It was a solo effort that analyzed the petitioners' political donations, their co-authorship on papers, and their connections to various nonprofits or companies. Mashey suggested that the petition was intended "to create and maintain doubt in the public" about the consensus on anthropogenic warming.

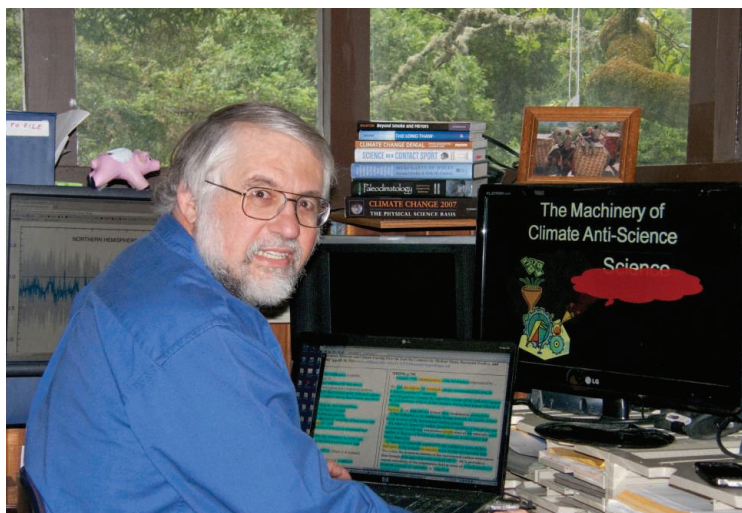
"For a long time, the amateurs in the online wars over climate science have been on the other side," Mann says. Those combatants include Steve McIntyre, a Canadian skeptic blogger and mining consultant. McIntyre's critique on the statistical analysis behind the so-called hockey stick (a graph by Mann showing that recent decades were warmer than any since 1400) was praised in the Wegman report and reported on the front page of *The Wall Street Journal*. Mashey, Mann says, is "the anti-Steve McIntyre."

While Mashey occasionally quotes experts attacking the central scientific arguments of his foes, he generally focuses on what he considers to be unacceptable professional behavior or connections to political or monied interests. As a result, his critics say, Mashey is more interested in destroying his foes than in debating the issues. Wegman told Elsevier in his e-mail that various investigations have made the year "a professional and personal nightmare."

Mashey says exposing poor scholarly practices is just as important as uncovering what he calls "bad science." The copied sections of the *CSDA* paper, he says, are "clear evidence of incompetence [that is] understandable to the public."

Mashey believes that vanquishing scientists' foes will serve a higher purpose. "It's up to some of the rest of us to help get these guys off your backs so you can do the science," he tells his scientist allies. He thinks discrediting their opponents also allows society to focus on the biggest problem: the need to reduce greenhouse gas emissions. "Goal number two is, try to help lessen the impact of climate antiscience on public policy before it commits the U.S. to be an increasingly bad place to live," he says.

—ELI KINTISCH



inconsistencies between testimonies and write them down, I likely have a big spreadsheet on one display, some Word document open to two places to compare, and several browser windows on several displays, as well as a bunch of PDFs open in [Adobe] Acrobat," he says.

His dense reports contain the fruits of his research and that of others, properly credited, as well as his opinions, in italics. An anonymous blogger dubbed Deep Climate first raised questions about the originality of material in Wegman's *CSDA* paper and his report to Congress, and Mashey included those examples and other questionable passages in his "Strange Scholarship" report, festooned with complex organizational charts and multicolored text.



# Mice Prompt Look at Cholesterol's Role in Female Fertility

It's easy to forget that cholesterol isn't simply a villain. The white, waxy substance is famously intertwined with heart disease, but cholesterol lives all over the body, building cell membranes and hormones. A small group of researchers is now wondering whether cholesterol also helps maintain fertility—and whether cholesterol abnormalities impair a woman's ability to get and stay pregnant. There's even speculation that a cholesterol-lowering drug, which is no longer on the market, could treat a subset of women who are battling infertility.

The complicated story starts more than 15 years ago, when Monty Krieger, a molecular geneticist at the Massachusetts Institute of Technology in Cambridge, discovered a cell membrane protein, scavenger receptor class B, type I (SR-BI). The receptor binds to high-density lipoproteins (HDL) and sucks out their lipids, clearing them from the blood. Unlike cholesterol-laden low-density lipoproteins (LDL), which can harm the arteries, HDL is generally considered the “good” cholesterol. Researchers theorize that that's because the particles remove cholesterol from arteries, and companies are even seeking drugs to raise HDL levels in the blood in order to prevent heart disease.

Krieger engineered mice to lack SR-BI, and as expected, they had high HDL cholesterol levels because the receptor wasn't available to facilitate their clearance. But the

mice had two surprising features: They had severe heart disease despite high HDLs, and the female rodents, but not the males, were also infertile.

Krieger set to work trying to solve both puzzles. The infertility puzzle also soon

***“Could women at an infertility clinic have a similar problem?”***

—ANNABELLE RODRIGUEZ,  
JOHNS HOPKINS UNIVERSITY

intrigued Annabelle Rodriguez, an endocrinologist at Johns Hopkins University in Baltimore, Maryland, who cares for adults with cholesterol disorders. “We said, ‘Could women at an infertility clinic have a similar problem?’” she recalls. The question was particularly compelling as Krieger had found that he was able to cure the infertility in the female mice with a cholesterol drug called probucol that lowers both LDL and HDL levels.

Admittedly, probucol is not the most promising drug to give to women trying to get pregnant: It was removed from the market in the 1980s because it causes a potentially fatal heart condition. Still, the idea that a simple drug might cure some infertility was so appealing that Rodriguez forged

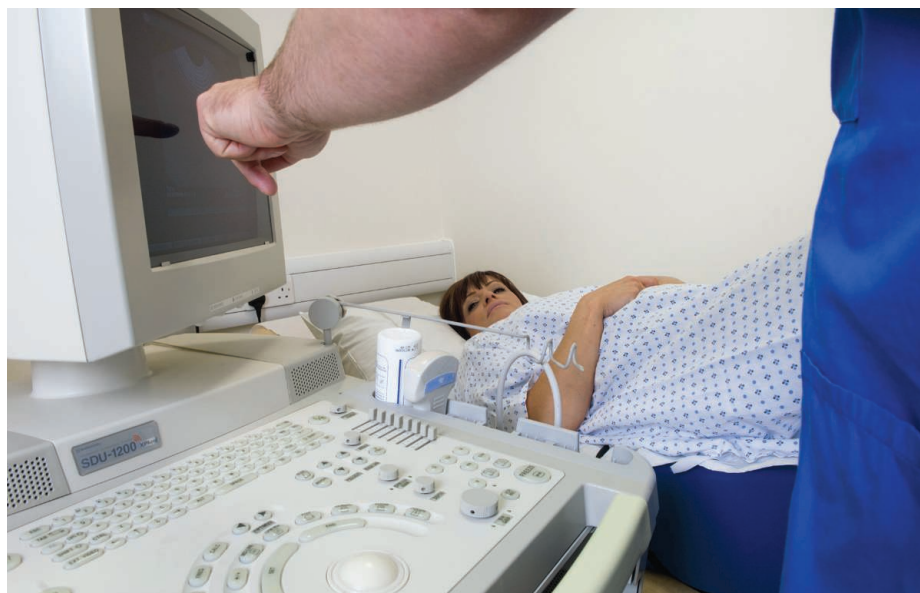
ahead to see if Krieger's story held up in humans. In 2009, Rodriguez found that people whose cells have fewer SR-BI proteins than normal tended to have higher HDL levels, suggesting that its function was similar in mice and humans. And last year, she

reported that the same receptor deficiency was associated with lower-than-average progesterone levels.

More recently, Rodriguez has turned to women coming to the Johns Hopkins in vitro fertilization (IVF) clinic because of fertility issues. There, she found that about 15% of the women tested had one or two copies of SR-BI gene variants previously associated with low production of the protein. More-

over, those women also had, on average, lower progesterone levels than women without the variants, according to Rodriguez's measurements of the hormone in the follicular fluid that surrounds a woman's egg. Nine women with one of the SR-BI variants were also unable to sustain a pregnancy after receiving their IVF embryo. In contrast, among those without that variant who had an embryo transfer, 207 women in all, about 30%, were able to get pregnant.

Rodriguez believes that the IVF clinic findings, published in *Human Reproduction* at the end of April, echo what Krieger saw in mice. Why would a dearth of production of SR-BI cause infertility? No one's really sure, but there are theories. An early one revolved around steroid hormones, like estrogen and progesterone, to which cholesterol is a precursor. Unlike some hormones, such as insulin, which the body stores and releases when needed, steroid hormones are made from scratch on demand, and cholesterol is the key ingredient. But Krieger found that in his mice lacking SR-BI, HDL cholesterol hangs around in the blood because it moves more slowly to other tissues. This could impair ovarian production of hormones such as estrogen and progesterone, which help control egg production or ready the lining of the uterus for implantation of a new embryo. Indeed, a study by a group in Montreal, published in April 2010 in the *Journal of Lipid Research*, found that mice lacking SR-BI had blood levels of progesterone about 50%



**New fertility player.** Cholesterol is increasingly being eyed for a role in helping women become or stay pregnant.

below normal. That's nearly identical to what Rodriguez observed in the follicular fluid of the women with the gene variants tied to poor SR-BI production.

Is the problem in mice and people too little progesterone due to too much HDL cholesterol in the blood? Krieger isn't convinced by the theory, noting that his mice had a normal menstrual cycle—something that's not possible without passable hormone levels. He has, however, found defects in the mouse eggs as they are ovulated and hypothesizes now that the problem, at least in mice, has something to do with cholesterol's impact on oocyte maturation. Rodriguez says she still favors the idea that progesterone is a problem for the women in her study, but she's stymied by limited information. In particular, she doesn't know the HDL levels of the women with SR-BI variants, because fertility clinics don't normally measure cholesterol, nor does she know if they had a normal menstrual cycle.

Muddling the picture further is a large family in southern Holland, many members of which were known to have high HDL levels. Those members have one mutated copy of the SR-BI gene, Jan Albert Kuivenhoven of the Academic Medical Center in Amsterdam and colleagues reported in January in *The New England Journal of Medicine*. Yet several of the affected women with a high HDL level nevertheless had children. "Fertility problems were certainly not in this family," Kuivenhoven says. Despite that, the lipidologist doesn't discount Rodriguez's work. The numbers in this family "are too small" to say anything definitive about fertility, he says.

Rodriguez says she has just submitted a grant application to the U.S. National Institutes of Health to study how SR-BI variants affect the menstrual cycle in women of child-bearing age, not just those undergoing IVF. She is also working on an investigational new drug application to test probucol in infertile women with SR-BI deficiency.

"We are just in the infancy of trying to understand" cholesterol's effects on fertility, says Victor Fujimoto, a reproductive endocrinologist at the University of California, San Francisco, who has found that a plentiful supply of HDL cholesterol helps keep IVF embryos in good health in the lab before they're transferred to women. Fujimoto acknowledges that exactly how this happens remains unclear, but whatever the answer, the broad-brush strokes are forming a clearer picture: Cholesterol seems to matter when it comes to fertility, at least in women.

—JENNIFER COUZIN-FRANKEL

**Testing, testing.** China is stepping up HIV testing, but a positive result can have serious social consequences.



CHINA

## Stigma of HIV Imperils Hard-Won Strides in Saving Lives

**BEIJING**—Encouraged by last month's news that AIDS mortality has plummeted in China, authorities here are embarking on a new 5-year plan for tackling the epidemic that includes ambitious targets for case detection and access to treatment. But further gains are jeopardized, critics warn, by rampant discrimination against HIV-infected individuals and the entrenched stigma of homosexuality in China.

"HIV/AIDS prevention is not just a clinical matter," says Joseph Lau, a public health expert at Chinese University of Hong Kong. "There are serious social and culture barriers to overcome to ultimately win the battle against the disease."

By the end of 2010, China had recorded 379,348 cases of HIV infection, including 138,288 people who had developed AIDS, and 72,616 deaths, according to Wu Zunyou, director of the National Center for AIDS/STD Control and Prevention of the Chinese Center for Disease Control and Prevention (CDC) here. After years of virtually ignoring the epidemic, China introduced antiretroviral therapy to treat HIV-infected people in 2002. That move made a huge difference: In *The Lancet Infectious Diseases* on 19 May, Chinese CDC researchers reported that AIDS mortality in China declined 64% between 2002 and 2009. Mortality rose slightly last year.

But for thousands of Chinese, interventions are coming too late, or not at all. CDC

estimates that there are 740,000 HIV-infected people—twice the reported number. Many AIDS patients in China are gravely ill by the time they are first tested for HIV, says Hao Yang, deputy director of disease prevention and control at China's health ministry. In 2010, a quarter of those who tested positive had already developed AIDS, and nearly half of the partners of HIV-infected people were infected; among fatalities, 82.4% died before getting treated. "Early detection remains the biggest challenge," Wu says.

In the new 5-year action plan, China aims to reduce new infections by 25% and the fatality rate by 30% by 2015, Wu says. To achieve those targets, the government will expand detection programs, aiming to diagnose 75,000 cases this year, up 16.6% from 2010, and put more than 40,000 more people on treatment; by the end of last year, 108,800 people were on antiretroviral drugs. Hospitals in regions with high infection rates intend to impose mandatory HIV testing for patients and for high-risk populations, including sex workers, injecting drug users, and men who have sex with men (MSM).

Many experts warn that the strategy is likely to backfire. "Mandatory HIV testing may pose threats to individuals' rights," says Zhang Konglai, an epidemiologist here at the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (CAMS). "There are reasons why people don't want to



go and get tested.” The chief fear is the black mark that HIV infection represents in Chinese society. “The consequence can be disastrous for people with HIV and their families if their HIV status becomes known,” says Guy Taylor, an advocacy expert in the Beijing office of the Joint United Nations Programme on HIV/AIDS (UNAIDS).

China’s top leaders have sought to ease the stigma. On World AIDS Day, state media have given prominent coverage to Chinese President Hu Jintao’s visits with AIDS patients. Hoping to reach out to more HIV-infected people, CDC has set up a network of some 9500 clinics that offer free and voluntary HIV/AIDS counseling and testing over the past decade.

However, few people avail themselves of these services. Many fear that a positive HIV status will not be kept confidential, Taylor says. According to a 2009 UNAIDS survey, nearly a third of HIV-infected individuals reported a breach of confidentiality by employers, friends, family members, and health care workers. The survey revealed that 12% of infected individuals had been denied treatment because of their HIV status, 7% had been forced to move home, and over three-quarters said that family members had experienced discrimination. More than one-third claimed that they had been refused a job, had been denied a promotion, or had their job duties changed as a result of their HIV status—despite a 2007 national law that guarantees HIV carriers the right to work.

Undermining that law are national regulations that prohibit people with HIV/AIDS from working in the civil service, or in hotels, cafes, and beauty salons, for example. Last August, a 22-year-old college graduate sued Anhui Province education bureau for rejecting his job application after he tested positive for HIV in a mandatory civil service blood test. He lost his case and the appeal. “This has sent a very bad signal and will encourage more employers to turn down people with HIV/AIDS,” says Zhai Xiaomei, director of CAMS’s Center for Bioethics here. With livelihoods at stake, few HIV-infected individuals are likely to submit to testing until they have full-blown AIDS.

In the meantime, HIV is spreading rapidly among MSM, who are estimated to account for a quarter of the infected population. In a 2009 survey of 61 cities in China, the infection rate among MSM was around 5%—100 times the reported national average—and up to 18% in places. “It’s just the tip of the iceberg. The infection rate may be much higher and is rising rapidly,” says An Ran, director of Shaanxi Tongkang Working Group, a non-



**No-nonsense message.** Billboard in Yunnan reads, “To prevent AIDS, men should act responsibly.”

governmental organization (NGO) in Xi’an that offers voluntary HIV/AIDS testing and counseling. Recent HIV testing in Xi’an bathhouses, a popular venue for MSM to meet, revealed infection rates as high as 50%. In Sichuan Province, Lau and his colleagues found that the HIV infection rate among MSM had risen from 1% in 2005 to 14% this year in the capital, Chengdu, and from 15% a couple of years ago to 23% in Zigong. There are an estimated 14 million or more MSM nationwide. According to Wu, 212,509 were tested for HIV in 2010; this year’s target is 236,600.

Complicating efforts to contain HIV’s spread, roughly a third of MSM in China are married, and half have both male and female sexual partners, says Zhang Beichuan, an AIDS expert at Qingdao University. “This is putting a large number of women at risk,” he says. “But HIV prevention targeting those women is an untouched subject in China.”

Another impediment to stemming HIV is the harsh treatment of injecting drug users. By the end of 2010, China had over 1 million heroin users, says Li Jianhua of the Yunnan Institute for Drug Abuse in Kunming. China has made inroads in this population. Some 700 methadone clinics and more than 1000 needle-exchange sites around the country have reduced HIV infection by half in the past decade among tens of thousands of visitors.

But many drug users “stay away from such services for fear of getting arrested,” Li says. In China, heroin users are detained in prison-like rehabilitation centers for up to 3 years. In these centers, which currently house some 230,000 drug users, detainees must submit to mandatory HIV tests, but often they are not told the results until just before release, Li says. With rare exceptions, he says, inmates

do not receive counseling or treatment.

With few signs that stigmatization is relenting, China must rely on NGOs to help reach out to HIV-infected people. To do so, Lau says, the government must ease restrictions on NGOs, which can register only if they are affiliated with a government agency; many operate without legal status or a bank account. Organizations that campaign for safe sex and public acceptance of HIV/AIDS and homosexuality report that they are often harassed by the police.

Last month, a simmering controversy over China’s reluctance to support genuine grassroots NGOs hit a boiling point when the Global Fund to Fight AIDS, Tuberculosis and Malaria halted grant payments to the country, citing concerns such as misuse of funds and corruption. In the current HIV/AIDS program, a 6-year, \$500 million scheme launched last year, China had pledged to allocate 20% of the first year’s budget to NGOs and gradually raise that to about 35%. “But most of the funding has gone to ‘official NGOs’ created and controlled by the government,” says Jia Ping, director of China Global Fund Watch, an NGO here. In response, the health ministry plans to overhaul its HIV/AIDS program; Minister Chen Zhu has vowed to correct spending irregularities and punish culprits.

With an estimated 50 million people at risk of HIV infection, China is at a critical stage in controlling the AIDS epidemic, Taylor says. “What we need is the normalization of HIV testing,” he says. “This is unlikely to happen without greater involvement of civil society ... or properly addressing the issues of stigma and discrimination.”

—JANE QIU

Jane Qiu is a writer in Beijing.

CREDIT: MALCOLM LINTON

## PLANETARY SCIENCE

# Planetary Two-Step Reshaped Solar System, Saved Earth?

Planetary scientists ponder a lot of questions about origins. Why didn't Mars grow as large as Earth and Venus? Where did the asteroid belt come from? What are Jupiter and Saturn doing so far from the sun? For that matter, why didn't Jupiter just drive Earth into the sun the way most Jupiter-like exoplanets have driven their rocky, Earth-sized neighbors into their stars? A new study that models the earliest solar system's gravitational fandango has an answer for each of those questions, and more. In the model, the solar system's fate is changed forever when Saturn snags the intruding Jupiter and together they back off before driving the still-growing Earth into oblivion.

Shuffling growing planets around "seems to work really well," says planetary dynamicist David Minton of the Southwest Research Institute (SwRI) in Boulder, Colorado, who was not involved in the work. "It seems crazy, but there are all these ways of moving planets around early in the solar system's history, when there was a lot going on." The new model may resolve decades-old debates about how the solar system evolved, Minton says.

The story starts 4.6 billion years ago, when the planets were starting to grow inside a sun-centered swirling disk of gas and 10- to 100-kilometer-wide planetesimals. In recent years, modelers of the early solar system have come to grips with two aspects of planetary behavior during that period—one fundamental, the other a bit of an oddity. Fundamentally, both modeling and exoplanetary observations show that as long as Jupiter and Saturn—the innermost and largest of our giant planets—were embedded in that protoplanetary disk, they would have been inexorably driven toward the sun. The reason was one of the unintuitive ways that gravity shaped the earliest solar system. Both Jupiter and Saturn had vacuumed their orbits clear of gas. But plenty more of it remained outside their orbits. Simulations showed that although unable to cross the gas-free gaps, the gas would, through gravitational interactions with the planets, inevitably push them inward as the gas naturally spread toward the sun.

The oddity is tiny Mars. It grew to only 11% the mass of Earth or Venus, its nearest neighbors. Planetary dynamicists could make an appropriately small Mars in a model but only if Mars started growing from

an inner disk of rock and gas that stretched no farther than 1 astronomical unit (AU) from the sun, the current distance of Earth's orbit. If Mars got nudged outside the disk, it would be starved of rocky planetesimals and grow no more.

What kept the rocky planet nursery confined to 1 AU? Planetary dynamicists Kevin Walsh, now at SwRI, Boulder, Alessandro Morbidelli of the University of Nice Sophia Antipolis in France, and colleagues wondered whether the culprit could have been an inward-migrating Jupiter. Although gravitational force is a pull, not a push, Jupiter could have in effect repulsed the planetesimals

1:2 resonance—would have to move, too.

So, as they report in this week's issue of *Nature*, Walsh and his colleagues set up a model of the earliest solar system—the first 5 million years—in which Jupiter migrates inward through a disk of gas and planetesimals to 1.5 AU. That would leave the rocky disk truncated at 1 AU, small enough to stunt Mars's growth.

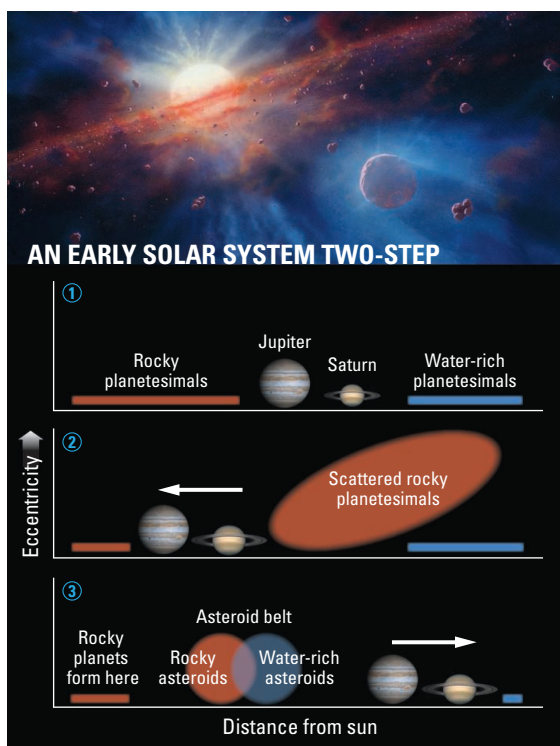
Why doesn't Jupiter keep going and destroy the inner disk entirely? As other modelers had shown, the faster-migrating Saturn eventually slips into a 2:3 resonance with Jupiter. That pairing lets pent-up gas slip through Jupiter's gap in the disk, freeing Jupiter of the gas's push. Thus unbound, Jupiter can ride outward, taking Saturn with it, as the more massive planet pushes off the gas nearer the sun by gravitationally raising tides in the gas. Within a few million years the disk's gas dissipates, and the planets settle down into stable orbits.

An inward-then-outward two-step does produce a small Mars. And it produces the right total mass for the rocky planets. Even more critically, it leaves a proper-looking asteroid belt in its wake. Jupiter and Saturn plow back and forth, scattering planetesimals—soon to be asteroids—as they go. Even under a range of initial conditions, roughly the right mass of asteroids ends up in a belt of rubble between Mars and Jupiter—just where they should be.

The model also produces a key detail of the asteroid belt: the distinctive predominance of dry, rocky asteroids in the inner belt and the prevalence of ice- and organics-rich asteroids in the outer belt. That match "gave us a lot of confidence" that their modeling was realistic, Walsh says.

"It's an exciting model," says planetary dynamicist William Bottke of SwRI, Boulder, who is not an author of the paper. "They could end up being right," but he still has reservations. For example, the researchers need to confirm that the model also gets the shapes and tilts of asteroid orbits right, Bottke says. "It's still not clear to me it's going to work out." Planetary physicist David Stevenson of the California Institute of Technology in Pasadena is also cautious but says "the fact you can get [the model] to do the right things is a major accomplishment." So whether Saturn was Earth's savior remains to be seen.

—RICHARD A. KERR



**In, out, all about.** From (1), the two giant planets moved inward (2), compressing the zone where Mars would form and scattering rocky planetesimals outward. Moving outward (3), they threw both sorts of planetesimals inward to form the asteroid belt.

inside its shrinking orbit, plowing them even closer to the sun. One way would be by locking them into an orbital "resonance." For example, a planetesimal closer to the sun might make two complete orbits in the time that Jupiter—being farther out—completes one. The two would repeatedly pass each other at the same point in their orbits, where Jupiter could each time tug strongly on the planetesimal. As Jupiter migrated inward into closer and closer orbits, the planetesimal—locked into the



# A Bengali Recipe For Disaster

**Under a thick blanket of mud in the Bengal Basin, the crust is twisting and crumpling. A major earthquake is inevitable. Can the region's teeming, shoddily built cities avert calamity?**

**DHAKA**—In the late afternoon of 12 June 1897 in Assam, northeastern India, the earth began to rumble. The eerie subterranean growl grew louder and louder and after a few minutes the ground began to shake, at first gently then with such violence that tombstones, masonry, and even people were flung into the air. The Great Assam Earthquake, estimated as high as magnitude 8.7, claimed 1626 lives. Several days later, a team from the Geological Survey of India set out to map the shattered land. “This was the first time someone had resurveyed after an earthquake,” says Philip England, a seismologist at the University of Oxford in the United Kingdom. Over months of wretched work in monsoon downpours, the surveyors measured an astounding 8-meter uplift on the northern edge of Assam’s Shillong Plateau, extending through the area that today is Bangladesh. Incredulous superiors dismissed the results as erroneous and buried the report.

The survey team would eventually be vindicated. And the more experts learn about the Great Assam Earthquake, the starker it stands as a warning to cities on the floodplains of South Asia, including Bangladesh’s densely populated capital, Dhaka. Perched on thick, alluvial sediments 200 kilometers south of the epicenter, Dhaka “was badly damaged” in 1897, says Syed Humayun Akhter, a seismologist at the University of Dhaka. The ground under much of the city

liquefied, destabilizing foundations. Thanks to the earthquake’s gradual buildup, most people in Dhaka managed to escape before buildings disintegrated. “It was a miracle there were so few deaths,” Akhter says.

Dhaka may not be so lucky next time. Scientists are finding that both its social features and its geology, including a hidden fault that seismologists believe is gathering stress beneath the sediments, could make the area more vulnerable than appreciated. Few structures in this city of 13 million are built to resist shaking. Even when the ground is quiet, Akhter says, “you sometimes read reports of buildings falling down.” Shoddy construc-



**Mud skipper.** Michael Steckler (center) helps lower instruments into a well in northern Bangladesh.

tion is prevalent across Bangladesh and many other developing nations in seismic danger zones. “It’s hard to see any realistic way to avoid the looming humanitarian disasters in places like this,” says Susan Hough, a seismologist with the U.S. Geological Survey in Pasadena, California. “The world is short on both resources and political will to make the necessary investments in risk mitigation.”

Among disasters waiting to happen, Bangladesh stands out for its unique geography, which is capable of transforming modest quakes into major calamities. A land of superlatives, the country is the world’s most densely populated—more than 150 million people occupy an area the size of Iowa—and is home to the biggest delta and one of the heaviest sediment fluxes. Each year the Ganges, Brahmaputra, and Meghna rivers carry about 1 billion tons of sediment from the Himalayas, depositing much of it in the Bengal Basin. These sediments, a couple dozen kilometers thick in places, amplify seismic waves and hide active faults. Last February, Akhter and colleagues from Columbia University’s Lamont-Doherty Earth Observatory in Palisades, New York, and other institutions embarked on a 5-year campaign to study the Ganges-Brahmaputra-Meghna delta. They will gauge the extent to which that heavy, wet blanket of sediment is ratcheting up seismic strain. They also hope to chart hidden faults, including what they call

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**Houses of cards.** Sections of Dhaka are rising on reclaimed land that's prone to liquefaction.

an “astounding geologic event”: They hypothesize that the eastern Himalayan front—an area of folding, crumpling, and mountain building driven by India's collision into Asia—is jumping forward to a new front 200 kilometers to the south. The new front is raising the crust of the Shillong Plateau above a blind fault under the sediment in Bangladesh.

Some potential dangers are already known. The Shillong Plateau's southern edge has not slipped recently and could uncork a magnitude-8 or greater earthquake. Closer to Dhaka, a magnitude-7.5 quake on the Madhupur fault would level 72,000 buildings and kill more than 130,000 people, according to estimates published by the Bangladesh government in 2009. The biggest threat of all lurks in the east and offshore in the Bay of Bengal: the northern stretch of the Chittagong-Myanmar-Sumatra plate boundary. On 26 December 2004, some 2000 kilometers south of Bangladesh, the Sumatra-Andaman earthquake—at magnitude 9.3, the third largest ever recorded—ruptured as much as 1500 kilometers of plate boundary, triggering tsunami waves that killed more than 230,000 people.

Bangladesh has been slow to come to terms with the seismic menace. “The nation is poor. This is reality,” Akhter says. But the government is strengthening the building code based on revised seismic maps, and its disaster agency is training 62,000 volunteers in earthquake response.

### Piling up the stress

When the Geological Survey of India team struck out into the field in the summer of 1897, they were venturing into uncharted



**Ferretting out faults.** Syed Humayun Akhter hopes to uncover evidence of buried geology.

territory. Although the surveyors felt aftershocks, they did not realize that the entire plateau was still moving as they gauged its dimensions. As a standard accuracy check, the team would measure a triangle, at least 10 kilometers per side. By the time the third angle of a given triangle had been measured, days or weeks after the first, “all the angles had changed by an amount they had never hitherto encountered,” says seismologist Roger Bilham of the University of Colorado, Boulder. Supervisors derided what they deemed to be sloppy work. They were wrong, Bilham says: Shillong Plateau was adjusting to many thousands of aftershocks.

Re-examining the data a century later in 2001, Bilham and England calculated that in the space of a few seconds, the Great Assam Earthquake had thrust up a 110-kilometer-long piece of Shillong about 10 meters. The crust beneath the plateau is pinched between massive forces: the Indian Plate, crawling

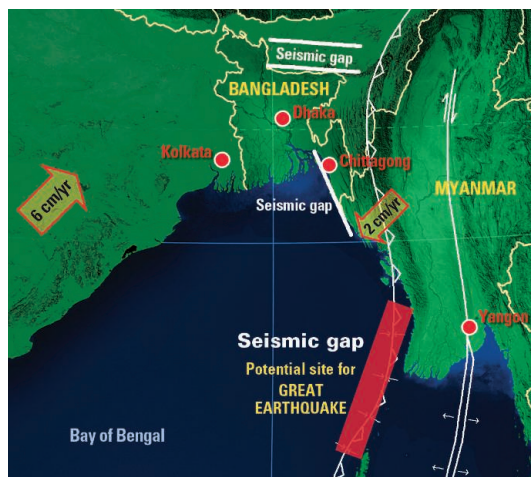
northeast toward the Himalayas at a rate of 6 centimeters per year, and the Burma Plate, grinding westward at 2 centimeters per year. When enough stress accumulates—perhaps every 3000 years or so—the plateau pops up, Bilham and England discovered.

The seismic waves from the rupture of the northern fault in 1897 rippled across the Ganges-Brahmaputra-Meghna delta, jiggling alluvial sediments like a bowl of pudding. Hough and colleagues have calculated that the shaking was two to four times more intense in Dhaka and other cities built on sediments than on settlements a similar distance from the epicenter built on rock. This amplification “has been shown to significantly control shaking and damage in countless earthquakes, including the 1811–1812 New Madrid earthquakes,” Hough says. Because most terrain in Bangladesh is soft sediments, any local earthquake measuring magnitude 5 or more would be punishing, Akhter says.

Another hazard is that when sediments are moist, they are primed to liquefy during an earthquake. Liquefaction of soil churned coast tracts on New Zealand's South Island during the magnitude-6.1 earthquake on 22 February, undermining foundations and leaving cars half-buried in sand, Hough says. In the Bengal Basin, during summer monsoon rains, the Ganges, Brahmaputra, and Meghna rivers deposit a sediment layer up to several centimeters thick across the delta. Surface flooding and groundwater recharge seasonally pile up 100 billion tons or more of water to the delta. The tremendous pressure can nudge a fault on the brink of rupture over the edge. “Most devastating earthquakes in this region have occurred during monsoon time,” including the 1897 Assam quake, Akhter says.

Soft sediments are not uniformly bad news. Like a car's shock absorbers, they can soak up strain from faults. It takes more time for stress to build in faults overlain by sediments, and thus the period between large earthquakes is longer.

To better understand how the Ganges-Brahmaputra-Meghna delta's thick sediments influence underlying faults, Akhter and colleagues have launched an ambitious research campaign. They will drill around 250 scientific wells to probe sedimentation and tectonics, gauge vertical shortening at two sites where well sets have been



**Grim picture.** New studies are shedding light on the Bengal Basin's complex tectonics. Most worrisome to geophysicists are the eerily calm Arakan and Tripura segments of the Chittagong-Myanmar-Sumatra plate boundary.



strung with optical fibers, measure electrical resistance of sediments, and use high-resolution seismic reflection to map sediments and underlying crust structure. “We will attempt to directly measure the compaction of sediments in the delta—how they squish as new sediments are piled on top,” says Columbia geophysicist Michael Steckler, the project’s principal investigator. A GPS network will monitor strain and a seismic network will record earthquakes, while rock outcroppings near Shillong and along the Chittagong coast in the southeast will be analyzed for evidence of past earthquakes.

### Planning for the worst

On 26 December 2004, in the hours after the Sumatra-Andaman earthquake, Akhter watched at the University of Dhaka as aftershocks marched up the plate boundary toward Bangladesh. “People were extremely tense and frightened,” he recalls. University officials called an emergency meeting that evening on how to cope in the event that the northern segment of the plate boundary were to rupture. According to campus policy, dormitory doors were locked at midnight. “We needed to change this without creating panic,” Akhter says. Resident teachers were asked to discreetly unlock the doors.

Although a northern rupture never materialized, the threat hasn’t gone away. The last major earthquake at that end of the fault occurred on 2 April 1762, along the Chittagong coast. The rupture of the Arakan fault segment, estimated to have exceeded magnitude 8, submerged islands just off the coast, triggered mud volcanoes, and caused seiches, inland tsunami waves, that capsized boats on the Buriganga River in Dhaka. Since then, Akhter says, a 600-kilometer stretch of the subduction fault from Myanmar to the Andaman Islands has been a seismic gap: in other words, strangely calm. There are no known ruptures of the onshore Tripura segment, north of Arakan. “We have no idea how much strain is building up in these segments,” Akhter says.

The subduction fault, he and others believe, is due for a major temblor. The Arakan segment would surely unleash a tsunami. Cyclones occasionally score a direct hit on Bangladesh, and the country’s low-lying, funnel-shaped coastline makes storm surges all the more deadly. Cyclone shelters dotting the coastline in principle could offer a haven from tsunami waves. But a tsunami would

make landfall within an hour after a quake on the Arakan or Tripura segments. “We would not be able to send a warning in time,” Akhter says. A major quake on the Tripura segment, meanwhile, would deal a crippling blow to Dhaka and much of Bangladesh. “This is the segment that most worries me,” Steckler says.

The biggest hazard in Bangladesh may be poor construction. Before Bangladesh’s independence in 1971, Akhter says, most buildings had at most three stories. Since then, to accommodate a constant influx of migrant workers, landlords have added floors to many buildings helter-skelter without shoring up foundations, he says. And new housing developments in Dhaka are rising on unconsolidated sediments augmented by fill—a recipe for liquefaction. There’s little the cash-strapped country can do about existing structures, Akhter says. But he hopes that new building projects will incorporate earthquake-resistant design. “We are now trying to convince architects and builders that they have to follow building codes,” he says.

Adhering to the current Bangladesh National Building Code may not offer sufficient protection. It is based on a seismic zone map prepared by the Geological Survey of Bangladesh in 1979. The zones were drawn up “on very scanty data,” Akhter says. He calls up a map of the zones on his laptop; a small area of northeast Bangladesh is designated as high risk. “To me now, this whole area should be high risk, including Dhaka,” Akhter says, tracing out the eastern half of the country, anchored by the Tripura fault segment.

The picture is similar in neighboring countries in a seismic belt stretching from northern India across Nepal and Bhutan and down through Bangladesh and Myanmar. The 26 January 2001 earthquake that leveled Gujarat, India, killing 20,000 people, happened in a region long known to be at high risk for shocks, Bilham says. But the code was so unevenly applied, he says, that the same percentage of the population was killed in collapsed buildings in 2001 as in an earthquake

in 1819—when earthquake-resistant construction was unknown.

“There’s no magic wand that will make a vulnerable city in the developing world safe,” Hough says. Bilham argues that it is essential to replace the ancient building stock of cities. That would substantially reduce the carnage, he says, and it would “make politicians and urban planners look less culpable than they do now.” It would take decades to gird cities in the developing world against earthquakes. In the meantime, Hough says, cash-strapped nations can take discrete steps. One “realistic goal,” she says, is to shore up critical facilities like hospitals, schools, and airports. Toward that end, politicians need advice, and pressure, from experts. “One of the most important things the international community can do is to help train and support local communities of earthquake professionals,” Hough says.

The task is daunting. “Many decades will pass before the developing world can hope to approach the levels of resilience that are potentially achievable,” England and James Jackson of the University of Cambridge in the United Kingdom warn in this month’s issue of *Nature Geoscience*. “During this time millions of people will be affected by earthquakes on faults that have not been recognized,” they write. The highest scientific priority, they argue, is to map with precision where the level of seismic hazard is increasing in the regions of greatest risk: the 10 million square kilometers of the Alpine-Himalayan belt, which stretches from Italy, Greece, and Turkey, across the Middle East, Iran, and central Asia, to India, China, and, of course, Bangladesh.

Back in his office, Akhter calls up a map of Bangladesh with red dots indicating major earthquakes. From 1869 to 1930, five temblors with a magnitude of seven or higher rattled the country. “It’s been 80 years since the last big earthquake,” he says. In that time, Dhaka’s population has grown 10-fold.

The perils are incalculable.

—RICHARD STONE



**Seismic sleuth.** Columbia geologist Leonardo Seeber inspects river sediment layers and a rare intact rock (right) in northern Bangladesh.





**Liftoff.** An icy plume with solar halo blasts from a fissure on Saturn's moon Enceladus.

## PLANETARY SCIENCE

# Enceladus Now Looks Wet, So It May Be ALIVE!

**Accumulating evidence from a Saturn orbiter points to liquid water in a distant moon, but further investigation of this likely habitable zone may be out of reach**

Liquid water—and the life it may permit—has been the goal of planetary exploration for decades, with not much of the sloshy stuff to show for the effort. Jupiter's moon Europa has a global ocean, but unfortunately, it's out of reach beneath many kilometers of ice. So the sight in 2005 of ice and water vapor jetting hundreds of kilometers above Saturn's icy little moon Enceladus, like Yellowstone geysers gone ballistic, warmed the hearts of astrobiologists everywhere. But as terrestrial geologists soon pointed out, water plumes needn't mean liquid water. Enceladus might be frozen solid and still be spouting water ice and vapor.

With new observations, the "Enceladus: Oasis or Iceball?" debate is now coming down on the side of a wet interior for the moon—and a chance for life. "Everything seems to point to some amount of liquid water," says cosmochemist Jonathan Lunine of the University of Arizona, Tucson. "It's hard to argue for a completely frozen moon."

The science team operating instruments on the Cassini Saturn orbiter had suggested that subsurface liquid water was boiling to form the plumes. But Susan Kieffer of the University of Illinois, Urbana-Champaign, a geological fluid dynamicist who has studied Yellowstone's Old Faithful geyser, saw a problem (*Science*, 15 December 2006, p. 1668). The Cassini mass spectrometer showed that the plumes contained considerable amounts of carbon dioxide, nitrogen, and methane, far more nitrogen and methane than could have been dissolved in liq-

uid water. Kieffer noted that only clathrates, water ice with various gas molecules trapped in the "cages" of its own crystal structure, could deliver so much of the gases once heating decomposes the clathrates. Enceladus could be solid, lifeless ice throughout.

Since Kieffer's 2006 challenge, Cassini team members have been accumulating more evidence for liquid water from more and closer flybys of Enceladus. In 2009, planetary scientist Frank Postberg of the University of Heidelberg in Germany and his colleagues reported having detected sodium salts in 6% of the particles that make up Saturn's faint outer E ring, which is created by the plumes (*Science*, 23 January 2009, p. 458). The composition of the ring particles resembled that of seawater, so Postberg and his colleagues argued that water from a deep ocean was reaching the fractures of the south pole region, where the plumes originate, and blasting salty droplets into space.

In May, at a meeting of the Enceladus Focus Group, Postberg and his colleagues reported that an extra-close flyby through the densest parts of the plumes had detected sodium salts in particles that account for more than 95% of the mass of particles. Liquid water, not solid clathrates, looks like the best bet for producing salty plume particles (although clathrates could still be producing the nitrogen, carbon dioxide, and methane).

Cassini infrared measurements of the temperature of Enceladus's plume-emitting south polar region are also pointing more strongly

toward liquid water. Last March, planetary scientists Carly Howett and John Spencer of Southwest Research Institute in Boulder, Colorado, and their colleagues reported in the *Journal of Geophysical Research-Planets* that improved observations had almost doubled the best estimate of the emitted heat energy to 15.8 gigawatts. "That's quite a lot of power," Spencer says. It's enough to drive liquid-derived plumes, which require higher subsurface temperatures than clathrates do. "Once you have temperatures that high, you don't need clathrates as a driving mechanism" for the plumes, Spencer says.

The great abundance of heat energy also points to liquid water. The only known way Enceladus could generate heat is for Saturn to raise tides in solid parts of the moon the way Earth's moon raises tides in the oceans. Repeated tidal flexing of Enceladus's ice would produce heat the way repeatedly bending a paper clip makes it warm. But Enceladus's interior would be too stiff for tidal heating to work if it were solid ice throughout, notes planetary physicist David Stevenson of the California Institute of Technology in Pasadena. A layer of ice overlying a sea of water would be flexible enough, he says. So would ice over a layer of water-filled cracks.

All in all, liquid water in Enceladus is "pretty likely," "most likely," or "almost incapable," scientists told *Science*. Most would agree with Spencer that the chances for liquid water are something like 90%. And all agree that at least some clathrates could be decomposing in order to explain the gases, especially methane.

Despite the general agreement, Cassini's set of instruments is unlikely to clinch the case for liquid water. Enceladus advocates are calling for another, more capable, mission to finish the job. Liquid, salty water laced with organic compounds "points strongly to a habitable zone beneath the south polar terrain," Carolyn Porco of the Space Science Institute in Boulder, who leads the Cassini camera team and co-organized the Focus Group meeting, writes in an e-mail. And whatever is inside the moon is continually ejected for close inspection. "So, Enceladus is THE most promising place for investigation of things astrobological," she writes.

But the spotlight has been on Jupiter's moon Europa so long (*Science*, 19 December 2008, p. 1780) that a mission to Enceladus was little more than an afterthought in March's Planetary Science Decadal Survey, which recommended a Europa mission (*Science*, 11 March, p. 1254). Moving Enceladus up the priority list would be a daunting task, liquid water or not. **—RICHARD A. KERR**





## ARCHAEOLOGY

## South African Cave Slowly Shares Secrets of Human Culture

In the hands of a skilled archaeologist, a South African site serves as a laboratory for testing ideas about early human culture and cognition

**SIBUDU CAVE, SOUTH AFRICA**—“Did you remember to put your insect repellent on?” It’s a question archaeologist Lyn Wadley asks of every visitor to her excavations at Sibudu Cave, chiseled into a cliff face high above the Tongati River in South Africa’s KwaZulu-Natal province. To get to Sibudu, Wadley and her team members have to drive down a dusty dirt road, wade across the knee-deep, swiftly flowing river, hike through tick-infested brush along the river bank, and scramble up a steep slope strewn with loose rocks to the ledge of this rock shelter, located about 40 kilometers north of Durban.

There on a broad, flat rock shelf, under the shade of a towering Natal elm tree, lies what has kept Wadley coming back to Sibudu for the past 12 years: an 8-meter-thick mound, a record of prehistoric human occupation that extends back at least 77,000 years and probably much longer. These multilayered, human-made sediments, which cover most of the 55-by-19-meter cave floor, are crammed with thousands of artifacts left behind by *Homo sapiens* during our species’ formative years, culturally speaking. There are sophisticated stone tools, skillfully made bone implements, deep hearths, and the charred bones of large and small mammals; there are swatches of bedding made of sedges and

grass, chunks of red ochre, and sparkling ornamental beads made from the shells of sea snails.

Under Wadley’s trowel, this site has become a powerful tool for testing hypotheses about the cognitive prowess of early modern humans. The excavations “have provided a powerful database,” says archaeologist Paola Villa of the University of Colorado, Boulder. For example, Wadley, of the University of the Witwatersrand (Wits), Johannesburg, and her team have pushed back the first signposts for what she calls “complex cognition,” producing evidence for the earliest known bows and arrows as well as the precocious use of snares and traps to catch



**Prime location.** Sibudu is a “perfect place” to study very early human culture.

small animals, both of which require the ability to plan ahead.

A stream of papers from Wadley’s team about early *H. sapiens*’ activities at Sibudu has made her one of archaeology’s most influential theorists and thrust Sibudu into the archaeological limelight. “Sibudu is probably the most important site in southern Africa at the moment,” says archaeologist Alex MacKay of Australian National University in Acton.

And yet the next chapter in the site’s history won’t include Wadley: She retired from fieldwork at the end of the spring field season. But given Sibudu’s importance—and the fact that Wadley has dug through only 3 of 8 meters of human occupation—the dig will go on. Wadley has passed the torch to a new crew chief, Nicholas Conard of the University of Tübingen in Germany, to uncover the rest of Sibudu’s riches, which may stretch back to 100,000 years ago or more. Wadley could have taken a peek at those earlier layers; other archaeologists urged her to dig a small, deep test pit to see what was there. But she decided against this shortcut, because then she wouldn’t have context for the oldest layers. “How would I make sense of it without knowing what was in between?”

### The road to Sibudu

The hominins who repeatedly visited Sibudu may have been drawn by its protective setting and an abundance of flowing water, fruit trees, and animals. But for Wadley, 64, the road to Sibudu was long and winding. She grew up in a small town in what is now Zimbabwe, an only child who loved to read, write, and paint, and she attended a teacher’s college in Bulawayo. There she was inspired to become an archaeologist, but she spent many years teaching before finally earning a Ph.D. in archaeology from Wits.

Wadley began to explore the Middle Stone Age (MSA), which ranges from about 300,000 to 40,000 years ago and includes Sibudu and other well-known South African cave sites such as Blombos. She started out working at Rose Cottage Cave, an MSA site on South Africa’s Caledon River, where she began to formulate her ideas about “complex cognition.” This concept, Wadley insists, “is a lot more empirical” and testable than the more commonly used notion of “modern human behavior,” because complex cognition focuses on actual cognitive tasks, such as toolmaking or hunting

strategies, rather than on vague notions of modernity and symbolism.

After 11 years working at Rose Cottage Cave, Wadley had an “extremely large and excellent” stone tool collection but no organic preservation of animal bones or plant materials that could tell her more about how the people lived.

So she began looking for a site that could better test her ideas about the technological abilities of early modern humans. Tipped off by another archaeologist who had briefly excavated the top layers of Sibudu, Wadley visited the rock shelter in 1998 and saw its thick MSA layers and excellent organic preservation. “I knew this was going to be good even before I put my trowel into the earth for the first time.”

Wadley and her team headed to Sibudu that same season, and as the years went by, they encountered, as she puts it, one “pleasant surprise” after another. So far, the team has determined that Sibudu was occupied intermittently between 38,000 and at least 77,000 years ago. “It’s a long and very complete sequence of occupation,” says new dig leader Conard. “No other site has a better record of human activity.”

This extremely long occupation record includes two sophisticated stone tool industries found across southern Africa, called the Still Bay and the Howieson’s Poort and dated to about 71,000 to 72,000 years ago and 60,000 to 65,000 years ago, respectively (*Science*, 6 May, p. 658). Wadley and her team have microscopically analyzed residues on the Howieson’s Poort tools and uncovered important clues about how they were used. Archaeologist Marlize Lombard of the University of Johannesburg found traces of tree gum and wood on the tools. That, along with patterns of wear that suggest the tools were not used for cutting or scraping, led her to conclude that they had been hafted to handles of wood or bone and probably used for hunting.

And in a paper published last year in the journal *Antiquity*, Lombard went even further, suggesting from the wear patterns and other evidence that some tools from layers dated to 64,000 years ago might have been used as arrowheads. That’s the earliest claim for bow-and-arrow hunting, which other archaeologists have argued was key to the success of our species, because it allows humans to take down large animals from a distance with less risk to themselves. “I find their paper quite convincing,” says anthropologist Alison Brooks of George Washington University in Washington, D.C.

**Making it stick.** Traces of red ochre on stone tools like this one may have been used to haft the tools to handles.



### Making art, or making glue?

Meanwhile, Wadley had been noticing that some Howieson’s Poort segments had traces of ochre on the edges that apparently had been hafted into wood or bone handles. She launched a series of experiments in which she mixed *Acacia* tree gums, beeswax, and ochre—which is also known to have good adhesive properties—in various combinations. She found that when she got the recipe right, the mixture had all the properties needed to tightly haft tools to handles.

In a widely read 2009 paper in the *Proceedings of the National Academy of Sciences*, Wadley and her colleagues detailed the multiple steps involved in this procedure: getting the ingredients ready ahead of time, heating them to the right temperature, and holding the entire hafting plan in one’s mind while carrying it out over time. They argued that this was better evidence for abstract rea-

abstract designs etched onto ochre pieces at Blombos 75,000 years ago.

Wynn says he is even more convinced by Wadley’s contention that the hominins at Sibudu probably used snares and traps to catch small animals at least 65,000 years ago. In a 2010 paper in the *Journal of Human Evolution*, Wadley argued that archaeological sites containing a high proportion of animals that are difficult to capture in any other way present circumstantial evidence of the use of snares and traps, which do not preserve well.

Indeed, the faunal record at Sibudu is dominated by small, forest-dwelling mammals such as a tiny antelope called the blue

duiker and the very aggressive bushpig, animals not easily hunted but vulnerable to falling into human-laid traps. Wadley

## Online

sciencemag.org

Podcast interview with author Michael Balter.

notes that recent hunter-gatherer populations in Africa use snares and traps to catch similar animals. If early *H. sapiens* at Sibudu were using snares and traps, Wynn says, “this would be strong evidence” for advanced cognition, because it would require planning over a span of several days and inhibiting the impulse to grab whatever food was immediately at hand.

Yet despite the progress Wadley and her team have made at Sibudu, there are many unanswered questions. Why did early humans return again and again to the site, leaving behind such a thick mound of evidence for their presence? What was so special about this place? The team has uncovered strong evidence that the hominins who frequented the cave stayed for extended periods, including traces of bedding made from sedges and grass. “They even ate breakfast in bed,” Wadley says, citing the numerous animal bones found among the plant remains.

It will now fall to Conard to answer those questions, if he can. Conard says “it was hard to say no” when Wadley asked him to take over the site: “Sibudu is a perfect place to study how human behavior evolved during the MSA.”

Even when retired, Wadley says she will never stop thinking about the hominins who returned repeatedly to Sibudu. Perhaps the artist in her is speaking when she says: “I like to imagine that they would stand here and look at the river and the lovely forest around it and think this was a very beautiful place. And since they had language, they would say to each other, ‘When the fruits next appear on the trees, let’s meet here again.’”

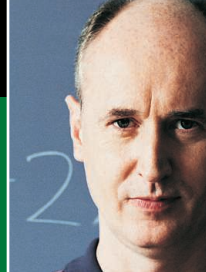
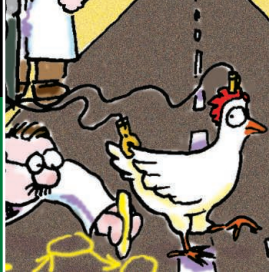
—MICHAEL BALTER



**A woman and her cave.** Lyn Wadley has spent 12 years excavating at Sibudu.

soning and advanced cognition than some other indications of symbolic behavior, such as the mere presence of ochre itself. “Lyn did a great job discovering and describing the steps involved in hafting [segments] onto shafts,” says archaeologist Thomas Wynn of the University of Colorado, Colorado Springs, who agrees that this process is a sign of advanced cognition. “But this needs to be weighed against the evidence that ochre probably had other roles,” he adds, citing





## LETTERS

edited by Jennifer Sills

## Taking Experimental Philosophy to the People

ARE THE IMPLICATIONS OF EXPERIMENTAL PHILOSOPHY LIMITED TO RESOLVING PHILOSOPHICAL issues? Are there no practical implications for, say, the legal system, or, even more broadly, how we understand ourselves and others to be responsible (or not) for our actions in daily life?

My greatest hope for philosophy is that it can effect change beyond the academy—that the greatest critical minds can motivate societal reflections and actions in a way that, regrettably, pop culture does not. My greatest reassurance that this is possible, thus far, has been S. Nichols' Review on experimental philosophy ("Experimental philosophy and the problem of free will," 18 March, p. 1401). Yet I fear that the promising field of experimental philosophy will unnecessarily limit its scope to dialogue among philosophers. Someone must play Socrates and take philosophy to the people.

Practical applications of philosophical issues abound. For example, knowing that one's intensity of emotion is proportional to one's bias of judgment could help when we lose our tempers. Knowing that people think differently about the causality of psychological states than they do about neural firings could inform our approach toward investigating social perceptions of mental illness. If people do not understand how to reconcile determinism and free will, then perhaps we should not challenge inconsistent thinking but instead highlight the paradoxes of causality that exist already within science.

I hope that experimental philosophy will seize the opportunity to make philosophical inquiry matter to a wider audience, in a direct, tangible, and relevant way.

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Experimental Philosophy:  
Surveys Alone Won't Fly

THE NEW FIELD OF EXPERIMENTAL PHILOSOPHY, reviewed by S. Nichols ("Experimental philosophy and the problem of free will," Review, 18 March, p. 1401), is based on the realization that arguments are better served by empirical data than by appeals to intuition. This is a welcome development. Unfortunately, experimental philosophers' methods, as described in the Review and at the experimental philosophy conference in New York (1), change the problem's scale rather than resolve it.

Experimental philosophers ask non-philosophers how they would react in a given

scenario. Surveys such as these, however, are an extremely limited research method. They are vulnerable to response biases, phrasing ambiguities, and most important, the well-established fact that people have very little conscious insight into the factors underlying their behavior, and little ability to predict how they will feel or act in novel situations (2).

For these reasons, experimental psychologists collect data by observing behavior in a novel situation, rather than just conducting surveys. Designing successful observation experiments requires rigorous controls for the above-mentioned biases and ambigu-

ities. Experimental psychologists have spent the past century developing ways to do this, and experimental philosophers should take advantage of those insights. Not doing so cannot be justified by calling the enterprise a new discipline.

For experimental philosophy scenarios that are too unrealistic to be constructed in practice, researchers should work to find adequate approximations. Virtual environments may be one solution. Such worlds frequently diverge substantially from reality,

even at the level of basic physics (such as Second Life, in which everyone can fly). Experimental philosophers could harness these environments to create impossible scenarios for actual experiments.

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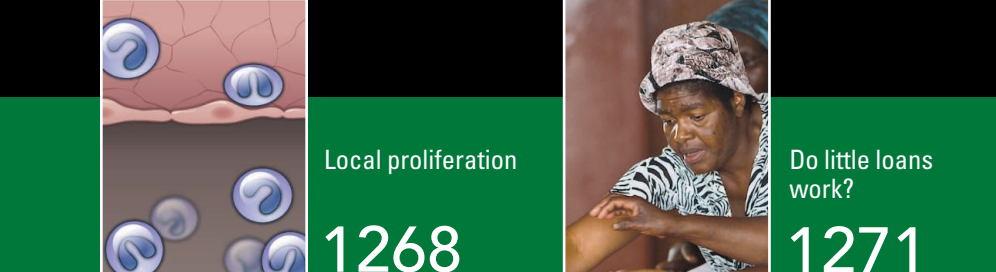
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2. J. A. Bargh, E. Morsella, *Perspect. Psychol. Sci.* 3, 73 (2008).

## Response

AS CARMEL NOTES, SURVEY TECHNIQUES have important limitations as a source of data. However, surveys are the most direct means to investigate ordinary reactions to philosophical issues. When we want to know whether people think that their choices are determined, it is hard to justify not consulting them directly. Experimental philosophers are not alone in using survey techniques; surveys continue to be widely used in many





of the social sciences.

Survey techniques are especially important when our interests are in content domains, such as the psychology of moral judgment, the cognitive science of religion, and research on political affiliation. Just as psychologists combine survey results with data from other approaches, such as reaction time and brain imaging, experimental philosophers embrace multiple techniques. We welcome Carmel's suggestion to examine behavior in new situations, but this strategy doesn't obviate the need for survey research.

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## Dealing with Data: Governments Records

IN HIS PERSPECTIVE "ENSURING THE DATA-RICH future of the social sciences" (special section on Dealing with Data, 11 February, p. 719), G. King discusses the potential to transform the future of social science using existing "huge quantities of digital information about people." As King states, the business sector has proven that powerful informatics applied to data about people can support better decision-making and foster innovation.

The same could be done to tackle difficult social problems once we figure out how to assemble and share large data sets while protecting privacy. King suggests building data archives to facilitate reuse of data. Large data archives already exist on survey data (1–5), but little attention has been given to government administrative data, which is a key source of information about our society. From the day we are born until we die, most of our activities leave traces in various government databases. Indeed, these government information systems continuously generate data about all aspects of our society, much like the satellites we use to monitor our physical surroundings.

Unfortunately, most administrative data are left to languish in legacy databases after their original use. We must invest in efforts to build a systematic pipeline to extract data

from these systems for secondary analysis. A well-integrated federated data system of administrative databases updated on an ongoing basis could hold a collective representation of our society. Designed, built, and properly managed by experts under appropriate protocols and oversight, such a data infrastructure could transform the social sciences and still protect individual privacy, just as well-maintained satellite data have transformed astronomy. A secure data infrastructure is critical for government decision support systems, transparency and accountability in government, and ultimately computational social science research. The Census Bureau (5) and others (6, 7) have already demonstrated the enormous potential of federated administrative data systems.

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## Science Title Misstep

THE TITLE OF THE 6 MAY NEWS OF THE WEEK story "At long last, Gravity Probe B satellite proves Einstein right" (p. 649) made me cringe. I find myself frequently repeating to students and the public that science doesn't "prove" theories. Scientific measurements can only disprove theories or be consistent with them. Any theory that is consistent with

measurements could be disproved by a future measurement. I wouldn't have expected *Science* magazine, of all places, to say a theory was "proved."

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## Response

Bennett is completely correct. It's an important conceptual point, and we blew it.

COLIN NORMAN

News Editor

## CORRECTIONS AND CLARIFICATIONS

**Policy Forum:** "Toward the second commitment period of the Kyoto Protocol" by A. J. Weaver (13 May, p. 795). The color legend label should read "Emissions ( $\text{kg}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )."

**Reports:** "Normalization for sparse encoding of odors by a wide-field interneuron," by M. Papadopoulou *et al.* (6 May, p. 721). On Fig. 2D (top right), the last letter of the words "odor" and "stimulation" (that is, r and n, respectively) was cropped. The PDF available online has been corrected.

**Reports:** "Relationship between clinical signs and transmission of an infectious disease and the implications for control" by B. Charleston *et al.* (6 May, p. 726). The third complete sentence on p. 727, which reads, "Additionally, when we used proxy measures of infectiousness the latent period appeared longer than the incubation period [whereas the transmission data suggested it was shorter (Fig. 2, A and B)]" is incorrect. It should read, "Additionally, when we used proxy measures of infectiousness the incubation period appeared longer than the latent period [whereas the transmission data suggested it was shorter (Fig. 2, A and B)]."

**Reports:** "Selective, nickel-catalyzed hydrogenolysis of aryl ethers" by A. G. Sergeev and J. F. Hartwig (22 April, p. 439). There were two errors introduced during proofs. The third sentence of the first paragraph should read, "In addition, brown coal's polymeric network contains aromatic C-O bonds inherited from lignocellulosic biomass, and the hydrogenolysis of these bonds could facilitate the liquefaction of this carbon source and its conversion to arene feedstocks (6)." The last sentence of the last paragraph should read, "Although these mechanistic issues have yet to be resolved, and turnover numbers of the catalyst must be improved, the results from this work have demonstrated that the selective cleavage of aromatic C-O bonds in the presence of aliphatic C-O bonds can be conducted without reduction of the arene units by using a widely available metal and the cheap, mild, and atom-economical reductant hydrogen."

## Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to [www.submit2science.org](http://www.submit2science.org).



## ENVIRONMENT

# A Roadmap to Nature's Benefits

Heather M. Leslie

The taste of fresh strawberries. The sounds of the surf and kids playing on a packed shoreline. Warm sun on your back as you kayak across the bay. These visceral summer experiences, just ahead for many of us, are what *Natural Capital* is all about: How does natural capital—or ecosystems and the many species and processes that are part of them—generate benefits for people? And, vitally, how will management, climate change, and other perturbations influence the provision of these benefits in the future? Through careful analysis and illustrative case studies, *Natural Capital* demonstrates how explicit consideration of these benefits, along with how and where they are produced, will enable us to more proactively and effectively sustain the world's ecosystems and the human communities that rely on them.

The science and application of ecosystem services (that is, the benefits provided by functioning ecosystems) have advanced tremendously since Gretchen Daily and colleagues presented a preliminary assessment of their value (1). *Natural Capital* captures the substantial progress of the past 15 years from theory to user-driven scholarship in an easily digestible yet impressive array of chapters that will be of interest to the seasoned ecologist or economist, the dedicated congressional staffer, and the conservation practitioner alike.

The book begins with a primer on the science of ecosystem services, which highlights why and how ecosystem services are being applied in diverse contexts, including China, Colombia, and the United States. The initial chapters also provide readers with a sense of three fundamental elements of ecosystem service assessment: quantifying the supply of services generated by ecosystems (How much? Where on the landscape?), estimating their value (What are they worth, in dollars or other common currencies?), and finally, assessing how they may change in response to management and other landscape-level changes (What are the trade-offs among services? Where are the synergies among conservation and development goals?).

Ecosystem service science, which is

largely drawn from ecology and economics, enables us to link ecosystem state and processes (such as rates of primary productivity and decomposition) with the generation of services that people value (such as the protection from coastal storms provided by salt marshes and other coastal habitats). This distinction between processes and services highlights the importance of “mapping” services explicitly: If no one is living along a particular stretch of coast, then the marsh there does not provide a coastal protection value (although it may well offer other benefits, such as carbon sequestration, recreation areas, and fisheries nursery habitats). The largest section of the book (occupying more than half its length) presents a complementary set of “multi-tiered models for ecosystem services” including, for example, freshwater provision, forest products, and nature tourism. Although the editors state up front that these chapters are meant to stand alone, given the 106 contributors, the vol-



**Source of many services.** In addition to the shellfish this Colombian woman is gathering to sell, mangrove forests provide fuel wood, storm protection, nursery habitats for fish, carbon sequestration, and nutrient cycling.

ume is remarkably coherent, both in terms of the overall development of themes as well as terminology.

Importantly, the volume is not intended only for conservation scientists and practitioners. As several contributors note, even though only one of the United Nations Millennium Development Goals (2) explicitly mentions the environment, fulfilling all eight of these international goals aimed at cutting global poverty will require healthy ecosystems.

The book's final chapters focus on how ecosystem services have been or could be incorporated into policy and management decisions. One chapter discusses evaluations of alternative future scenarios for land use in Oregon's Willamette Basin and in a watershed on the Hawaiian island of Oahu. Other case

studies in this section include links between poverty and the environment in Kenya and the Amazon basin, the valuation of mangrove ecosystems in Thailand, and benefits provided by nearshore marine ecosystems in the Caribbean and Puget Sound, Washington. These chapters are perhaps the most interesting, as they illustrate the many challenges we will need to address if the ecosystem services approach is to be widely adopted. For example, incorporating adaptive behavior by resource users, governments, and other actors into ecosystem service assessments requires information and technical capacity that is at present often impossible to achieve. Yet capturing these dynamics, along with other feedbacks between social and ecological systems, is vital. Other challenges include determining appropriate policy mechanisms (e.g., cap and trade, payment schemes, or taxes), tailoring them to local institutional contexts, and simply getting this idea that humans depend on ecosystems in fundamental, diverse ways on the agendas of more government and civil society institutions.

Although *Natural Capital* does an excellent job of documenting technical advances in the field, action-oriented readers may wish that the interspersed short essays, often focused on model applications and other real-world perspectives, received more space. Hopefully a future book will pick up where this one leaves off. For the time being, those interested in incorporating ecosystem service approaches in their decision-making can find some helpful tools online [e.g., (3)].

## Natural Capital

Theory and Practice of Mapping Ecosystem Services

Peter Kareiva, Heather Tallis, Taylor H. Ricketts, Gretchen C. Daily, and Stephen Polasky, Eds.

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To date, ecosystem service projects have occurred primarily in terrestrial environments, and most have been led by governments or large environmental organizations. We likely will see a greater diversity of ecosystems and institutions involved in the next wave of ecosystem service assessment and management. For example, ecosystem services are a centerpiece of the new U.S. National Ocean Policy, and some companies (such as Dow Chemical, in partnership with the Nature Conservancy) are embracing ecosystem services as a means to assess potential business strategies. By providing a roadmap for how to move from well-grounded theory to real-world practice, *Natural Capital* offers an excellent resource for these and many other emerging ecosystem service projects. Yet in order for these efforts—and this volume—to fulfill their promise, scholars and practitioners will need to continue to work together to assess how ecosystem services are likely to change in an uncertain future, particularly in the face of humanity's adaptation to our changing environment.

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## NEUROSCIENCE

# Why We Laugh

Walter Sinnott-Armstrong

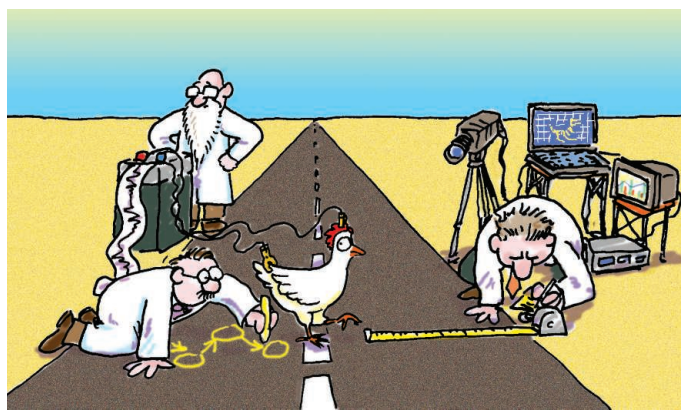
*Inside Jokes* is funny in two ways. It is funny ha-ha, because it includes hilarious jokes. (Question: "How do you tell the sex of a chromosome?" Answer: "Pull down its genes.") It is not funny huh, because it is clear and accessible rather than confusing or disturbing. Yet it is funny peculiar. Like a funny-looking tree, it holds your interest and raises new questions.

Science advances by asking new questions, and in the book, Matthew Hurley, Daniel Dennett, and Reginald Adams raise a lot of them: Why is humor enjoyable? Why do we laugh out loud? Why do we tell jokes? Why do we go to such great lengths to hear jokes? Why are some jokes funny and others not? Among funny jokes, what makes some funnier than others? Why does humor depend

on knowledge and culture? Why do some old jokes (and movies) still make us die laughing, whereas other jokes (and puns) die when repeated? Why are timing and order so important when telling jokes? Why is humor always about humans and never about rocks or roses unless they are anthropomorphized? What makes caricatures, nonsense, and incongruity funny? Why are our own failings and foibles funny? How does first-person humor differ from third-person humor? Why are practical jokes funny? Why is it funny to disparage certain groups of people but not others? How do sexual innuendo and bathroom references enhance humor? Why do men get more laughs and women give more laughs? Why do we laugh—and laugh so long—when tickled? Why don't other animals have a sense of humor? Why is the sense of humor so widespread in humans? Some of these questions have been asked before, but no previous attempt succeeds in answering so many so well.

The key to the authors' success is that they locate humor within recent cognitive science and evolutionary theory. To aid survival, our brains constantly and covertly use heuristics to generate expectations about what we will experience next, but we would be too inventive for our own good if we did not regularly search for and remove discrepancies between our expectations and our experiences. The immediate incentive to look for such discrepancies and thereby to reduce error comes from the pleasure of discovering a mistake in a currently harmless active belief that was introduced covertly. That pleasure is mirth, and humor is what produces it. Thus, humor is "a cognitive cleanup mechanism" that stains with mistaken belief before washing out the error (as in "I wondered why the Frisbee was getting bigger, and then it hit me."). Laughter is then a public signal of our ability to clean up our minds. Because such cognitive prowess is useful, it attracts mates—both friends and sexual partners—and spreads throughout the world.

Hurley, Dennett, and Adams apply their theory to well over a hundred examples



(including stupid jokes, dark humor, musical jokes, and witty remarks that are not humorous), to many apparent counterexamples (such as surprises, forgetting, riddles, and lies), and to related phenomena (such as magicians and garden-path sentences). They also use their theory to answer or refine the questions above, as when they explain why jokes can be ruined by taking too little time (for an active expectation to form) or too much time (so that the active belief is given up before the punch line) even though theatrical comedies can last for hours. Analysis is said to ruin jokes, but I still found myself laughing while the authors carefully dissected joke after joke. Perhaps my reaction could be taken to support their theory.

Their account also suggests several larger lessons. Humor is related to the scientific method of testing hypotheses, although it operates more quickly and covertly. Humor also illustrates how epistemic emotions motivate and control cognition. Reason is the slave of the funny bone. And humor exemplifies the human tendency to project our emotions onto the world, since humor is a feature of our thought processes rather than of jokes or any other external stimuli in themselves. Because humor is central to and distinctive of human life, understanding it can help us understand much more about ourselves.

Many parts of the authors' theory are, of course, not new. Hurley, Dennett, and Adams laugh on the shoulders of giants. In addition, much work remains to be done, especially regarding the neural mechanisms of humor. And critics will surely propose counterexamples and refinements. Whether or not the details of this theory survive, it is a very promising leap forward. Everyone who reads *Inside Jokes* will be enlightened as well as amused.

10.1126/science.1206802

#### Inside Jokes

Using Humor to Reverse-Engineer the Mind

by Matthew M. Hurley,  
Daniel C. Dennett,  
and Reginald B. Adams Jr.  
MIT Press, Cambridge, MA,  
2011. 373 pp. \$29.95, £22.95.  
ISBN 9780262015820.



## EDUCATION

# Preparing Future Math Teachers

William H. Schmidt,<sup>1,2\*</sup> Richard Houang,<sup>2</sup> Leland S. Cogan<sup>2</sup>

Poor precollege math abilities, and too little emphasis on college-level math, can reduce the number of highly capable math teachers.

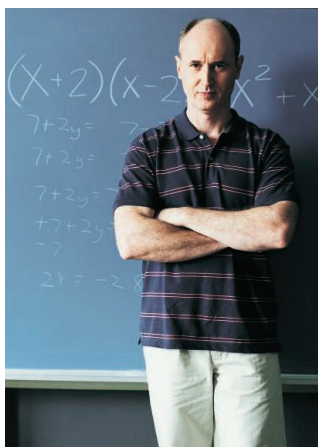
The U.S. President's Council of Advisors on Science and Technology recommends that the federal government provide support over the next decade to recruit and train at least 100,000 new science, technology, engineering, and mathematics (STEM) teachers of middle school (ages 11 to 13) and high school. Their strong academic backgrounds should include both "deep content knowledge in STEM subjects and mastery of the pedagogical skills required to teach these subjects well" (1).

How can this be accomplished for middle school mathematics teachers? What is the role for recruitment versus what must be accomplished through preparation? In part, the answer requires an understanding of what a well-qualified middle school mathematics teacher is. This has been a puzzle for over 40 years (2). That teachers need to have "some" knowledge of mathematics is always mentioned, but there is little agreement as to how much. Some have suggested that a strong background is sufficient, as evidenced by proposals to place college-educated individuals with degrees in mathematics in the classroom without any or with very limited teacher training (3, 4).

We address this issue by reexamining data from the 2010 Teacher Education and Development Study in Mathematics (TEDS-M), a 16-country survey of math teachers-in-training near the end of their final semester. TEDS was conducted by the International Association for the Evaluation of Educational Achievement (IEA) with an eye to developing international benchmarks for teacher preparation. This is similar to what was done for K–12 (primary and secondary) curricula via the IEA Trends in International Mathematics and Science Study (TIMSS).

## TEDS-M Results

U.S. middle school mathematics teacher preparation does not produce teachers with an internationally competitive level of mathematics knowledge. U.S. future teachers' TEDS scores straddle the divide between countries whose middle school students do better than the United States on international tests such as TIMSS, and those who do not (5, 6). (The only exception is Malaysia, which outperformed the United States in TIMSS but fell below the United States on TEDS).



Who is best prepared to teach middle school mathematics?

This relatively weak TEDS performance suggests that teacher quality may be the Achilles heel, as more than 40 U.S. states move to implement the mathematics Common Core State Standards ([www.corestandards.org](http://www.corestandards.org)). Will teachers have the needed knowledge to implement these internationally benchmarked standards that are more rigorous than previous standards which were "a mile wide, inch deep" [p. 122 of (7)]?

TEDS also documented how future teachers were prepared, e.g., which courses were taken (8). Generally agreed-upon cognitive competencies necessary for teaching mathematics encompass: (i) mathematics knowledge, (ii) pedagogical knowledge related to the teaching of mathematics, and (iii) general pedagogical knowledge related to instructional practices and schooling more generally (9, 10).

Future middle school teachers in all TEDS countries provided course-taking data that allowed us to characterize the percent of teacher-preparation course work in each of the three areas. The two highest-achieving countries, Taiwan and the Russian Federation, had on average a ratio across the three areas of roughly 50%:30%:20%. Roughly half of students' teacher-relevant efforts were in mathematics. By contrast, the estimated ratio for the United States was about 40%:30%:30%. The percentage of coursework on mathematics pedagogy was the same, but the general

pedagogy emphasis was higher in the United States, which balanced out the lesser focus on formal mathematics (11).

## Who Enters Teaching?

The pool of K–12 graduates from which the United States obtains its future teachers is weak compared with their peers internationally. On average, U.S. future teachers as they enter teacher preparation programs have been exposed to a less-demanding K–12 curriculum and have lower levels of mathematics knowledge than those in other countries. This promotes a vicious cycle. Weak K–12 mathematics curricula taught by teachers with an inadequate mathematics background produce high school graduates who are similarly weak. Some graduates then become future teachers who are not given a strong mathematics preparation at the college level. They then teach, and the cycle continues.

TIMSS eighth-grade mathematics data can define the pool from which future teachers are drawn. This allows estimation of country selection effects, the relative level of mathematics knowledge with which a country's typical future teacher enters teacher preparation.

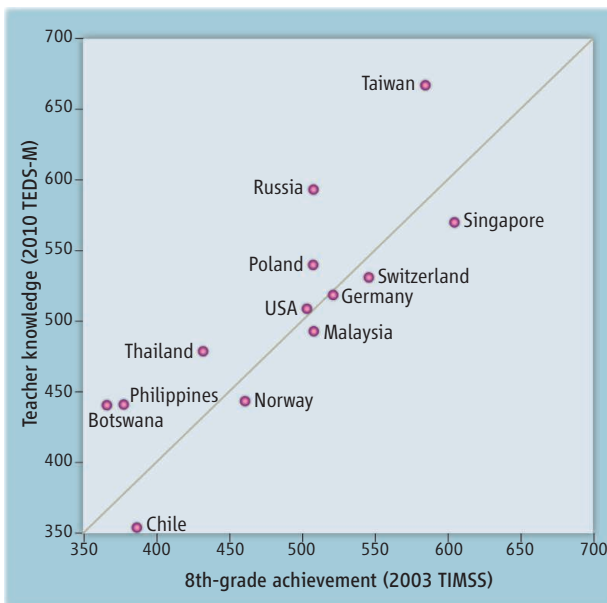
If Taiwan and Singapore were to select their average eighth graders (as represented by median performance on each country's distribution for the 2003 TIMSS) to become future middle school mathematics teachers, the United States would have to draw its future teachers from above their 75th percentile to be comparable to those from Taiwan and Singapore in their knowledge of mathematics (5). It is likely that, in some countries, future teachers are recruited from the upper end of the national distribution, through higher teacher salaries relative to other mathematics-oriented professions, which further exacerbate the differences (12). To obtain a well-prepared and well-qualified teaching force, any country must attend to who is recruited and selected.

## The Role of Preparation

Another way to explore this issue is to compare the knowledge of potential future middle school teachers of mathematics, as reflected by TEDS, with the average eighth-grade mathematics achievement, as found in TIMSS (13). The strong relation

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**Teacher preparation versus student scores in mathematics.** TEDS scores for future middle school mathematics teachers versus TIMSS scores for eighth graders. See SOM.

between TEDS and TIMSS [the square of the correlation coefficient ( $R^2$ ) = 0.70,  $P < 0.0004$ ] reflects how selection and/or recruitment based on knowledge before entry into teacher training relates to future teachers' knowledge when exiting from training (see the chart). Because both tests use the same scale, the diagonal line in the chart represents what would be observed if the mean TIMSS score for a country yielded the equivalent mean TEDS score (14). Some countries' mean TEDS scores are higher than would be expected from their mean TIMSS scores (above the diagonal line); others' are lower [supporting online material (SOM) (15)].

What might explain why countries fall above or below the line? Above-the-line countries may recruit future teachers from high school graduates who were in the upper end of their country's eighth-grade TIMSS distribution. Differences in course requirements of the preparation programs may also play a role. Every preparation program in every country included course work in each of the three broad areas described above. What differed was the relative emphasis given each area. Among those countries above the line, almost half (49%) of courses related to teacher preparation that were taken was devoted to mathematics, with only 21% emphasizing general pedagogy. For countries below the line, mathematics was given, on average, 37% emphasis with 28% emphasis on general pedagogy (see SOM).

To look more closely at the United States, each institution's average TEDS score was

plotted against the average SAT (a standardized college admissions achievement test) mathematics score for the institution's TEDS-participating students (16). Across the more than 80 randomly sampled public and private teacher preparation institutions, 56% were above the line and the rest were on or below the line. A pattern of coursework emphasis was found similar to that described for all TEDS countries. Future teachers in U.S. institutions above the line allocated, on average, about 40% to mathematics and 28% to general pedagogy. Below the line, the averages were about 30% for mathematics and 34% for general pedagogy. The 9% difference for mathematics courses was statistically significant ( $P < 0.0001$ ) (see SOM).

#### What to Do

The implication given the relatively weak U.S. K–12 mathematics curriculum is that recruitment of future teachers must come from the upper end of the U.S. distribution of mathematics performance in order to be somewhat competitive with high-achieving countries. However, this may not be feasible if current efforts to raise salaries for such individuals are not realized. In addition, the data summarized above also highlight the importance of emphasizing courses in mathematics in the preparation of such teachers.

Thus, the solution for the United States lies in a combination of recruiting those who have strong quantitative backgrounds together with a greater emphasis on rigorous mathematics in teacher preparation. The latter needs to be driven by tougher middle school mathematics teacher certification requirements, which are set by state policy. Perhaps it is time to consider something like a “Common Core” for teacher preparation, to provide more rigorous, demanding, internationally benchmarked preparation standards for mathematics teachers. This effort might be led by the National Governor's Association and the Council of Chief State School Officers, the same two groups responsible for the Common Core State Standards Initiative. A long-term and better solution is to break the cycle of mediocrity in which

we find ourselves. The Common Core State Standards are one promising new component aimed at improving student learning.

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14. The line was defined as equal scores on both measures, i.e., TIMSS and TEDS (see SOM).
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16. The SAT and TEDS scores were rescaled to have a mean of 0 and a standard deviation of 1. Where only scores on the American College Testing standardized college admissions exam were provided, these were converted to SAT equivalent scores. See SOM.

#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/332/6035/1266/DC1](http://www.sciencemag.org/cgi/content/full/332/6035/1266/DC1)

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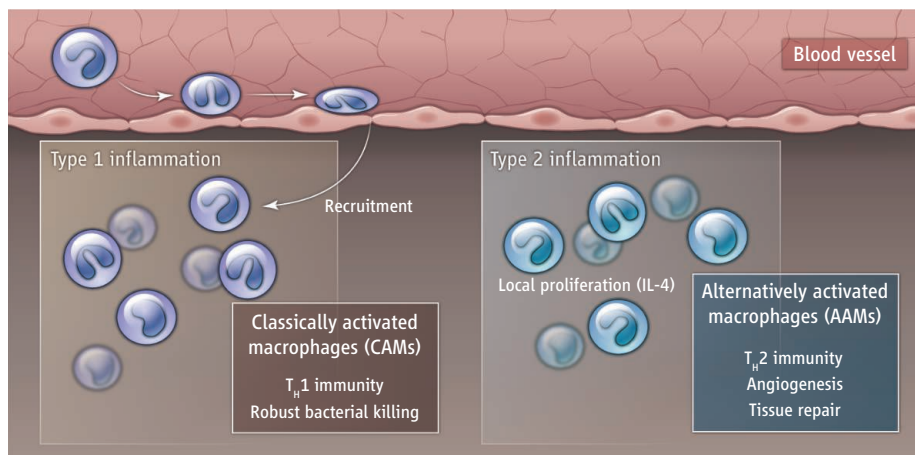
# No Need to Coax Monocytes

Gwendalyn J. Randolph

Although inflammation has been thought to rely on recruitment of macrophages from the blood, tissue macrophages can proliferate for an inflammatory response.

Macrophages are among the immune system's most important line of innate defense. They engulf and kill microorganisms and secrete factors that afford them key roles in tissue repair and remodeling. Macrophages are found in all resting tissues at low amounts; local demand dictates that their numbers increase in response to inflammatory signals. In many chronic diseases including heart disease, asthma, and cancer, inflammation overstates its welcome and drives disease. In many of these scenarios, accumulated macrophages are unwanted, and suppressing this build-up has therapeutic potential. Monocytes that circulate in the bloodstream are recruited to inflamed tissues and give rise to macrophages, and therapies to control inflammation often focus on interrupting this recruitment. However, on page 1284 of this issue, Jenkins *et al.* (1) reveal that the accumulation of certain macrophages—called M2 macrophages or alternatively activated macrophages (AAMs)—occurs not through coaxing their precursors from the bloodstream, but by local proliferation of macrophages (see the figure).

AAMs are generated *in vitro* by differentiating human blood monocytes in the presence of the cytokines interleukin-4 (IL-4) or IL-13, both of which signal through the receptor IL-4R $\alpha$ . Exposure to IL-4 gives rise to a distinct phenotype that differs from macrophages activated by classical macrophage-activating cytokines such as interferon- $\gamma$  (2). AAMs are typically associated with parasitic infections, humoral immune responses favored by T helper 2 (T<sub>H</sub>2)-type lymphocytes (hence the name M2 macrophage), angiogenesis, and wound healing. Their role in wound healing can be subverted to perpetuate pathologies such as fibrosis and tumor growth (2), but they may promote recovery from myocardial infarction and liver injury and stave off heart attacks by contributing to the stability of regressing atherosclerotic plaques (3–5). Classically activated macrophages (CAMs) are conversely associated with robust intracellular killing of bacteria and support of T<sub>H</sub>1-type lymphocytes. They are implicated in obesity and associated insulin resistance, whereas AAMs within adipose



**Macrophage accumulation.** AAMs collect at sites of inflammation in response to local proliferation, in contrast to CAMs, which are recruited from blood.

tissue preserve insulin sensitivity and suppress obesity (6). It is postulated that CAMs and AAMs are extremes on a wide spectrum of phenotypes (7). But studies *in vivo* validate the idea of polarization, in which a macrophage can exist in either an alternatively activated or classically activated state.

Two models offer differing explanations for the accumulation of AAMs versus CAMs at a given inflammatory site. One model stems from the fact that the same population of monocytes can be reversibly differentiated into one of the two fates depending on the cytokines present. In this scenario, polarized macrophages can be reprogrammed to the other polarization state by modulating the environment. In the other model, the two types of macrophages are proposed to derive from different subsets of circulating monocytes. Classical monocytes, which express Ly-6C in mice, are thought to be precursors for CAMs, and those lacking Ly-6C as precursors for AAMs (8). Each model assumes that blood monocytes are the source of both types of macrophages.

Jenkins *et al.* show in mice that AAMs that accumulate in response to helminth infection or IL-4 stimulation arise solely by local proliferation. Remarkably, no input from blood monocytes is required. Proliferation is driven by IL-4, the classical inducer of AAMs. Although homeostatic maintenance of resident macrophages and dendritic cells was shown to involve local proliferation (9, 10), this is the first example of inflammation,

and its associated accumulation of additional macrophages, achieved solely by proliferation. Not only is this mechanism unexpected, but it also widens the gap between CAMs (which the authors show to be dependent on monocyte recruitment) and AAMs. AAMs not only promote an inflammatory response to fight infection or promote wound healing, but they also clear apoptotic cells and other debris during the resolution of inflammation. Mechanisms that promote resolution of inflammation can limit the influx of leukocytes, including monocytes, from the blood (11, 12). Thus, local proliferation of AAMs provides the macrophages needed to promote resolution. Although unexpected, the mechanism is elegantly logical.

What of the notion that polarized macrophages can switch phenotypes? Jenkins *et al.* show that CAMs recruited from blood monocytes can be converted to proliferating AAMs by IL-4. Thus, macrophages at an inflammatory site can indeed be of mixed origin, arising from both a local and a blood-supplied source. However, in most of their experiments, the origin of AAMs was completely local. The authors assume that the increased number of AAMs is due to proliferation within the pool of resident macrophages, which in the absence of IL-4 exhibits a nonpolarized state. Perhaps other possibilities may apply. Tissue-resident hematopoietic stem cells can be coaxed during inflammation to give rise to macrophages (4, 13, 14). Although convincing, this has been perplexing, as it seems unnecessary to bolster

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the robust supply of monocytes that can be rapidly mobilized from blood. With the revelation that AAMs derive locally, the idea of a more primitive precursor for macrophages localized within tissues now takes on a greater semblance of logic. After liver injury, hematopoietic stem cell–derived macrophages assume an alternative, healing phenotype and boost tissue repair, even in mice with normal monocytes (4). Their natural residence in tissue may serve as a source of macrophages in IL-4–induced, proliferation-mediated inflammation. Thus, although Jenkins *et al.* cleverly break the mold of assuming that inflammatory macrophages must be recruited from the blood, they operate on an assumption that resident, nonpolarized macrophages are the

major source of AAMs in their studies. More investigation into this question will be needed.

A key future step is to determine whether proliferation accounts for AAM accumulation in human tissues and whether organs differ in the ability to give rise to AAMs in response to IL-4. If this applies in humans, the therapeutic implications are immense. Perhaps anti-proliferative drugs could be used to limit unwanted AAMs. Blockade of monocyte recruitment may now be envisioned to limit CAM accumulation in conditions such as obesity, without concerns that the approach would affect beneficial AAMs in the same tissues. The findings of Jenkins *et al.* challenge us to reevaluate some fundamental concepts about inflammation and how we should

target the macrophages that regulate it.

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10.1126/science.1208480

## CHEMISTRY

# Taking the High Road and Getting There Before You

Barry K. Carpenter

Advances in experimental or theoretical methodology sometimes reveal that the existing model for a phenomenon under study is actually incorrect or, at best, incomplete. One long-standing model that is being rethought in light of recent results is the transition state theory (TST) model, especially when applied to the selectivity of a chemical reaction—why more of one product forms versus another under a given set of conditions. In the conventional TST model, reactants must scale an energy barrier and pass through a transition state to form stable products. Reactions with lower barriers are usually the favored products. On page 1300 of this issue, Schreiner *et al.* (1) report that low-temperature reaction of an organic molecule is completely at odds with TST predictions, and that a proper accounting of the quantum-mechanical nature of the nuclei in molecules is needed to explain the results.

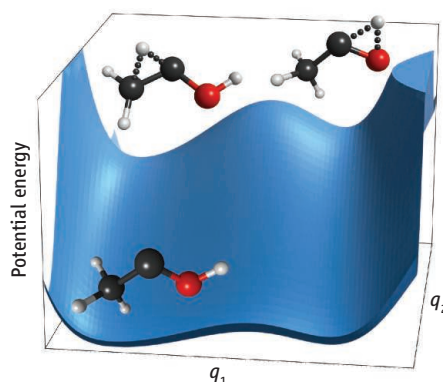
The selectivities of reactions under kinetic control (where the barriers for reverse reactions are high and prevent products from reforming reactants) can usually be described by applying TST to the potential energy surface (PES) that accounts for the energy barriers of the reaction. The PES is a multidimensional plot of the energy (usually the lowest possible energy) of the molecule as a function of the

coordinates  $q_i$  of its nuclei (which change as reactants collide with one another and when bonds break and form). The PES is calculated by applying the Born-Oppenheimer approximation, which assumes that the motions of electrons and nuclei in reacting molecules can be separated because of their very different masses. With this approximation, the motion of the nuclei can be described by classical mechanics, applying quantum mechanics only to the electrons. The full PES is difficult to compute and usually is visualized for most reactions by showing a projection with just one or two geometrical coordinates.

A schematic representation of such a PES projection is shown in the figure for the internal rearrangement reactions of methylhy-

Quantum-mechanical tunneling causes an organic molecule to follow the “wrong” reaction pathway with a higher energy barrier.

droxycarbene studied by Schreiner *et al.* This molecule has a divalent carbon atom that bears two nonbonded electrons. Like other carbenes, it is prone to reactions in which these electrons form bonds and return the hypovalent carbon to its more normal four-bonded state. Schreiner *et al.* generated methylhydroxycarbene in a matrix of solid argon to prevent it from undergoing bimolecular reactions. The only reaction pathways open to the isolated carbene are hydrogen migrations from either of the two different groups, –OH or –CH<sub>3</sub>, attached to the divalent carbon. The OH pathway generates acetaldehyde, whereas the CH<sub>3</sub> pathway generates vinyl alcohol. Schreiner *et al.* also performed sophisticated quantum-mechanical calcula-



**Through barriers, not over them.** A schematic is shown for the potential energy surface along two reaction coordinates,  $q_1$  and  $q_2$ , for the competitive hydrogen-atom migrations of methylhydroxycarbene studied by Schreiner *et al.*; the carbene is shown at the front of the diagram (hydrogen atoms are in white, carbon atoms are in black, and oxygen atoms are in red). The two transition structures for the competing reactions of the hydrogen atoms of the –OH group (right) or the –CH<sub>3</sub> group (left) are shown above their respective cols on the potential energy surface. At low temperatures, the favored pathway is unexpectedly through the higher barrier on the right that leads to acetaldehyde and is enabled by quantum-mechanical tunneling.

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tions and found that the barrier to the O–H migration was higher than to C–H migration.

The way in which TST converts PES parameters into predictions of product ratios can be viewed, qualitatively at least, as if the region around the reactant in the figure is a mountain basin that is filling up with water. The product ratio would then be related to the rate at which water flowed through each of the two gaps, or cols, that represent the transition states for the competing hydrogen migrations. If the widths of the cols (in the  $q_1$  direction) were comparable, the higher flow of water (the faster reaction) would be over the lower barrier. A higher barrier path could, in principle, be favored if its col happened to be much wider than the alternative, but in this particular reaction that was not the case. The clear prediction of TST to the computed PES is that the C–H migration should be the favored reaction, and vinyl alcohol the favored product.

The experimental reality was quite different: At 11 K in solid argon, the carbene exclusively produced acetaldehyde, and did so with a half-life of 66 min. Not only was the wrong product formed, there also should have been no detectable reaction of any kind given the calculated barrier heights and the low temperature.

The explanation for these seeming discrepancies comes from a breakdown of the Born-Oppenheimer approximation. In reality, the nuclei of atoms should also be treated as quantum-mechanical objects, meaning that they have simultaneous particle-like properties and wave-like properties; the wave-like extent of an atom is called its de Broglie wavelength. When reactions occur with atomic nuclei moving distances comparable to their de Broglie wavelengths, then the conventional picture of a PES breaks down. The de Broglie wavelength of a particle is inversely related to its mass, so migrations of hydrogen atoms are particularly susceptible to the effect.

Returning to our water analogy, it is as if the PES becomes porous, and so reaction can occur by leakage through the barriers as well as flowing over their tops. The efficiency of this leakage now depends on the width of each barrier (in the  $q_2$  direction in the figure) as well as its height, and for the reactions studied by Schreiner *et al.*, this was the key; the computed width of the barrier for the O–H migration was much smaller than that for C–H migration and made quantum-mechanical leakage much more efficient for the former reaction.

This discovery adds to the doubts about the general applicability of TST to predictions of selectivity. Other problems can also be summarized by appeal to the flowing-water analogy. Sometimes the “water” is flowing “downhill” on the PES and encounters a split in the available channels (2). Each branch leads downhill to a different product, but neither has a barrier. In these circumstances, conventional TST can make no prediction of the product ratio. In addition, examples have been found in which the initial direction of flow of the “water” matters. It is as if the basin in the figure were being filled with a fire hose pointed in the direction of one col. Under such circumstances, there may initially be greater flow over the barrier that is in the line of fire, even if it is the higher one (3). Together, these phenomena seem to be calling for a new model for chemical reactivity, but finding one that is both accurate and tractable will undoubtedly prove to be a challenge.

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## CELL SIGNALING

# New mTOR Targets Grb Attention

Sung Su Yea<sup>1</sup> and David A. Fruman<sup>2</sup>

A cellular enzyme called mechanistic (or mammalian) target of rapamycin (mTOR) controls cell growth and division, and is an important drug target in cancer (1). Despite extensive study, a complete understanding of mTOR function has remained elusive. One reason is that rapamycin, the natural compound that led to the identification of mTOR, only partially inhibits the enzyme. In addition, mTOR functions in two distinct protein complexes (mTORC1 and mTORC2). Furthermore, only a few proteins have been identified as mTOR substrates, and these seem insufficient to explain its myriad functions. Two papers in this issue, by Hsu *et al.* on page 1317 (2) and Yu *et al.* on page 1322 (3), uncover dozens of new substrates

and downstream targets of mTOR through proteomic screens. These results have major implications for research and drug development in cancer and metabolic disorders.

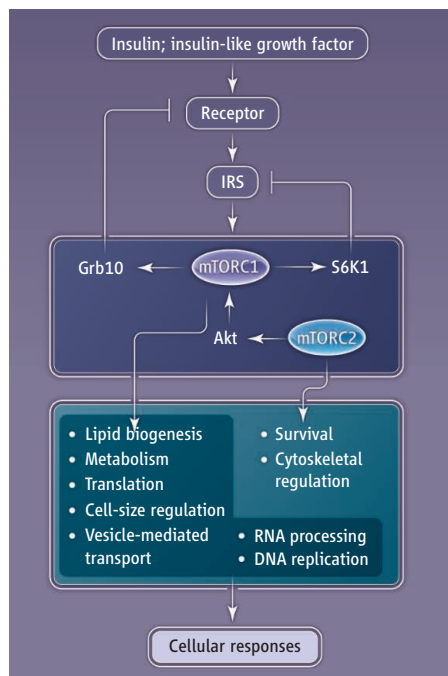
mTOR is a kinase that phosphorylates serine and threonine residues on target proteins. Both Hsu *et al.* and Yu *et al.* sought to identify all cellular proteins whose phosphorylation is directly or indirectly controlled by mTOR. Both studies used recently discovered mTOR kinase inhibitor (TOR-KI) compounds that are highly selective (4) and fully block the kinase activity of mTOR in mTORC1 and mTORC2. By contrast, rapamycin suppresses phosphorylation of only some mTORC1 substrates and is generally inactive toward mTORC2. Hsu *et al.* and Yu *et al.* each used two complementary approaches to stimulate mTOR activity in cells. One strategy involved the use of mammalian cells genetically modified to lack tuberous sclerosis complex 2, a negative reg-

The cellular phosphorylation targets of an important growth promoting enzyme are identified and may provide clinically relevant insights.

ulator of mTORC1. In the second approach, cells were deprived of growth factors before treatment with insulin, a factor that rapidly activates mTORC1 and mTORC2. Phosphorylated proteins were isolated and analyzed by mass spectrometry—a “phosphoproteomics” approach. As a complementary strategy, Hsu *et al.* defined the consensus phosphorylation sequence for the mTOR enzyme in vitro.

Although the details of the phosphoproteomic methodology differ between the two studies, the conclusions are remarkably similar. Both studies identify known mTOR substrates as well as hundreds of previously unknown protein phosphorylation sites controlled by the kinase. A large fraction of these known and novel substrates is insensitive to rapamycin. Further, Hsu *et al.* show that most of the rapamycin-sensitive phosphorylation sites do not fit the consensus amino acid sequence for direct mTOR substrates, suggesting that they are downstream targets in

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the signaling network. Together, these results help to explain why experimentation with rapamycin has uncovered few direct mTOR substrates. Also, the incomplete effect of rapamycin on the mTOR signaling network helps to clarify why rapamycin analogs have shown limited efficacy as anticancer drugs. The new direct mTOR substrates inferred from the phosphoproteomic data include several that function in processes not previously linked closely to mTOR including mRNA processing, DNA replication, and vesicle-mediated transport.

An important finding of both studies is that mTOR controls a large portion of the phosphorylation response to insulin, as most of the phosphorylation events stimulated by this factor were suppressed in cells treated with TOR-KIs. A clinical implication of this result is that treatments using TOR-KIs are likely to cause some insulin resistance. This validates previous findings showing that mTORC1 and mTORC2 together regulate most members of the AGC superfamily, which includes kinases involved in insulin signaling such as Akt and S6 kinase 1 (S6K1) (1). Many of the TOR-KI-sensitive sites do not conform to the consensus motifs of either mTOR or AGC kinases, which suggests that these kinases initiate signaling cascades that activate other protein kinases.

Hsu *et al.* and Yu *et al.* illustrate the biological impact of their data by focusing on the same candidate mTOR substrate, Grb10. This cytoplasmic protein suppresses signaling by insulin and the related insulin-like growth factors (IGFs). Mice lacking Grb10

**mTOR targets.** A proteomic approach has expanded our knowledge of the processes controlled by mTOR. A large portion of the cellular phosphorylation response to insulin is controlled by mTOR.

are larger than normal and exhibit enhanced insulin sensitivity (5, 6). Both studies show that Grb10 is directly phosphorylated by mTORC1, a modification that enhances Grb10 stability. Consistent with mouse genetic data, depletion of Grb10 from cells increased insulin sensitivity, whereas overexpression suppressed insulin signaling. Grb10 appears to participate in a negative feedback loop whereby mTORC1, activated downstream of insulin or IGFs, phosphorylates Grb10 and potentiates its ability to suppress continuing signals from insulin and IGF receptors (see the figure). This mechanism is complementary to a known feedback pathway in which S6K1 phosphorylates the insulin receptor substrate (IRS)—a protein that has a positive role in insulin and IGF signaling—targeting it for degradation.

It had long been suspected that the S6K1-IRS feedback loop was insufficient to explain the powerful negative control of insulin responsiveness by mTORC1; identification of the mTORC1-Grb10 mechanism thus fills an important gap in our knowledge. As Yu *et al.* describe, mining of transcriptome data pushes

the implications of this finding further by suggesting that Grb10 has tumor-suppressor function in human cancer. A related point of relevance to cancer research and treatment is that TOR-KIs are likely to enhance signaling through IGF receptors, which can support survival of cancer cells. However, TOR-KIs profoundly suppress downstream events in insulin and IGF signaling, which might mitigate the loss of negative feedback control.

Grb10 is just one example of an exciting lead generated by phosphoproteomic screens. Follow-up studies will likely yield new insights about mTOR biology and will hopefully provide clinical investigators with new biomarkers to monitor mTOR inhibitor action, along with improved understanding of their anticancer activity as well as toxicities to normal tissues.

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## ECONOMICS

# Why Finance Matters

Jonathan Morduch

A study from the Philippines adds to the case for rethinking the use and impact of microcredit loans.

**R**oughly one-half of the world's adults, about 2.5 billion people, have neither a bank account nor access to semi-formal financial services such as “microcredit,” the growing practice in developing nations of providing small loans, typically less than US\$500, to self-employed people (1). But what if they did? Muhammad Yunus, the 2006 Nobel Peace Prize winner and founder of Bangladesh's Grameen Bank, a pioneering microcredit institution, argues that this lack of financial access means that the poor, especially poor women, can't obtain the loans they need to build their businesses and get on a path out of poverty (2). The

idea has taken hold: In 2009, for instance, Grameen Bank served 8 million customers; its average loan balance was just \$127 (3). Worldwide, microcredit advocates now claim more than 190 million customers (4). Proof of concept, however, is not proof of impact. Recent studies have found that some efforts to provide small loans have produced surprisingly weak results, and on page 1278 of this issue, Karlan and Zinman (5) provide more evidence that we need to rethink microcredit. Their findings, from a randomized evaluation of microcredit lending in the Philippines, add to a handful of recent results (6–8) that suggest that microcredit's effectiveness has been overstated by studies that selectively focus on success stories.

To avoid this bias, researchers must carefully account for the fact that microcredit

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borrowers may be atypical members of their communities. They may be more entrepreneurial, for example, or better connected to markets, or more focused by nature. To create truly comparable groups, Karlan and Zinman worked with a lender to identify 1601 marginally credit-worthy applicants and then used specially designed software to randomly assign them to two groups: 1272 received loans; 329 did not. Then, they surveyed the participants over the next 11 to 22 months, asking them to report on such things as the success of their business, their borrowing habits, and their subjective well-being.

Karlan and Zinman first show that access to microcredit did indeed expand financial access (it didn't merely substitute for other lending possibilities, such as borrowing from a family member). Still, even with greater financial access, positive impacts on business were elusive. After roughly 1 to 2 years of borrowing, the household business activity of microcredit borrowers actually fell slightly relative to the nonborrowing control group (data on profit, which would be ideal for evaluating business success, were too noisy to be useful). And, on an index measuring subjective well-being, the scores of borrowers worsened.

These results matter because they are not isolated. The handful of other careful studies have also yielded mixed results that fall far short of the expectations set up by advocates of microcredit. None of the studies is perfect; Karlan and Zinman, for instance, work with a lender that does not focus on the poor, making an explicit evaluation of microcredit's role in poverty reduction impossible. But the studies deserve serious attention because they test microcredit while addressing selection bias so much more credibly than past studies. And the accumulating results should hasten efforts to rethink microcredit—not with the aim of dismissing it, but instead with the aim of reformulating our understanding of why people seek small loans, and how they actually use them. In essence, to appreciate the revolution that Yunus launched, it is necessary to think beyond Yunus.

The original vision of microcredit is enticing but too simple: Advocates have argued that the poor seek capital for business investment above all else. The idea that microcredit creates a new class of hard-working, indepen-



**Cash flow.** Women in South Africa meet to save and make small loans, motivated not just to build a business, but also to cope with the cost of such things as school fees, funerals, and unforeseen expenses.

dent entrepreneurs sells well with investors and donors. But it risks undermining support for what may be a more important reason to care about access to finance: that it helps families cope with economic challenges that may not be related to business.

We need to read between the lines to recognize this emerging concept. For example, it is important to consider that the microcredit offered by the bank studied by Karlan and Zinman is not cheap: The bank charges an annualized interest rate of 60% for loans with 3-month terms (Grameen Bank, by comparison, charges 20%). The unspoken question here is, Why are people taking business loans if gains to business are hard to detect? Perhaps one answer is that the borrowers are fooling themselves, imagining much bigger gains than they get. Or perhaps the positive impact of microcredit needs 3, 4, or more years to emerge, not the 1- to 2-year window that researchers investigate.

A different answer, however, emerges from new, small-scale descriptive studies of poor families (rather than from structured statistical evaluations)—as does, more importantly, a different question (9). These studies show that borrowers turn to microcredit for a range of uses beyond what they tell lenders. The loans help address health problems, for instance, and there are also school fees to pay, food to buy, and other (more expensive) loans to repay. A large study in Indonesia found that about half of the loans to poor borrowers went to purposes unrelated to business (10). A similar result holds in Bangladesh for a small sample of Grameen Bank borrowers (9). In the Philippines, a broad survey revealed that only 37% of major expenditures from microcredit loans went to business purposes (11). (The

survey asked only about expenditures larger than \$20; another 39% of spending was unaccounted for, presumably pertaining to expenses under \$20, some of which likely went to business.)

These results give us a very different frame for understanding microcredit. It emerges as a tool that largely helps families cope with ups and downs and make big-ticket purchases (some of which are related to business). The results suggest that having better ways to borrow will not, by itself, rid the world of poverty, but can provide stability and liquidity to families living near the edge. Managing money becomes vital when you have little of it.

This perspective agrees with an emerging body of research, from around the world, showing that the irregularity and insecurity of income is as challenging a problem for the poor as having low income (9). It also helps explain a pattern observed by Karlan and Zinman: Borrowers bought less formal insurance than nonborrowers, which suggests that they used microcredit loans as an alternative mechanism to cope with ups and downs. It is likely that helping poor families borrow and manage cash flow—reliably, flexibly, and for a range of uses—will end up being the truly big idea behind microcredit, even if it's not the big idea that inspired the global movement.

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# Terrestrial Ecosystem Responses to Species Gains and Losses

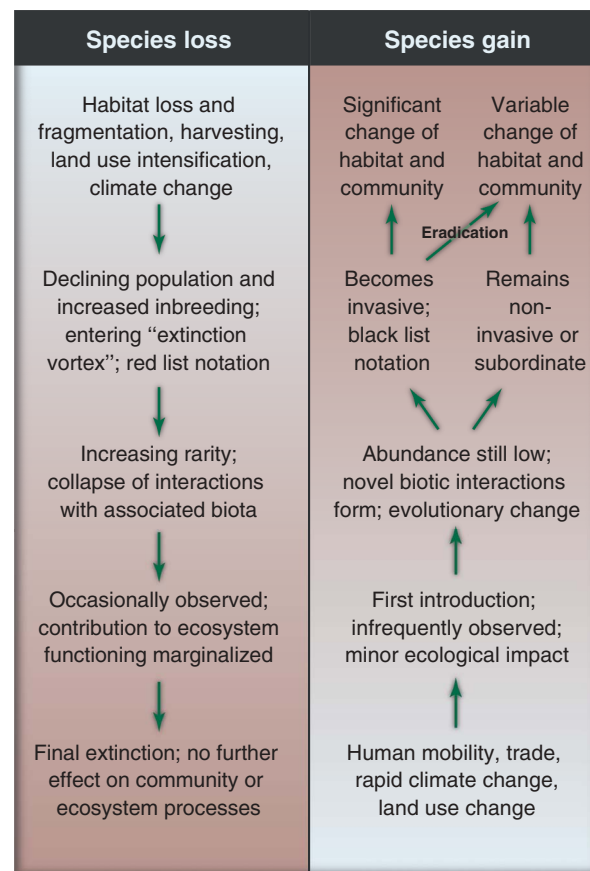
David A. Wardle,<sup>1\*</sup> Richard D. Bardgett,<sup>2</sup> Ragan M. Callaway,<sup>3</sup> Wim H. Van der Putten<sup>4</sup>

Ecosystems worldwide are losing some species and gaining others, resulting in an interchange of species that is having profound impacts on how these ecosystems function. However, research on the effects of species gains and losses has developed largely independently of one another. Recent conceptual advances regarding effects of species gain have arisen from studies that have unraveled the mechanistic basis of how invading species with novel traits alter biotic interactions and ecosystem processes. In contrast, studies on traits associated with species loss are fewer, and much remains unknown about how traits that predispose species to extinction affect ecological processes. Species gains and losses are both consequences and drivers of global change; thus, explicit integration of research on how both processes simultaneously affect ecosystem functioning is key to determining the response of the Earth system to current and future human activities.

**H**uman-induced global change is causing ecological communities to rapidly lose some species and gain others, resulting in interchanges of species, their traits and interactions, and alteration of ecosystem functioning and services. Substantial losses of species at both global and local scales have occurred as a consequence of human-induced factors (1) and are expected to continue well into this century (2, 3). Human migration and global change have also facilitated the spread of a vast range of organisms into new habitats, leading to biological invasions of many communities and often homogenization of species among them (4). Because local-scale losses of native species and ingress of new species occur simultaneously, both net gains and losses of species richness are occurring (5, 6). Although the Earth is experiencing substantial losses of biodiversity at the global level (1), both increases and decreases in community diversity are commonly observed at regional and local scales (5).

This interchange of biota is highly relevant to the growing interest in the role that species attributes have in driving terrestrial ecosystem processes, both above ground and below ground (6, 7). Species losses have their greatest effect when the lost species were previously abundant and/or had functionally irreplaceable roles. Further, the effects of new or invasive species are likely to be greatest when they possess functionally novel attributes that the native species lack

and/or when they become abundant in the recipient ecosystem. Despite gains and losses of species at local scales being comparable pro-



**Fig. 1.** The sequence of events generally associated with species loss and gain over time, revealing conceptual parallels and differences between the two processes.

cesses (Fig. 1), the literature on the effects of species gains on ecosystem properties has largely developed independently of that on the effects

of species losses. In this review, we bring these two issues together to develop insights into how ongoing and simultaneous gains and losses of species in terrestrial communities can cause shifts in the distribution of traits in the biota, thereby radically altering the functioning of terrestrial ecosystems.

## Species Gain: Invasion and Range Expansion

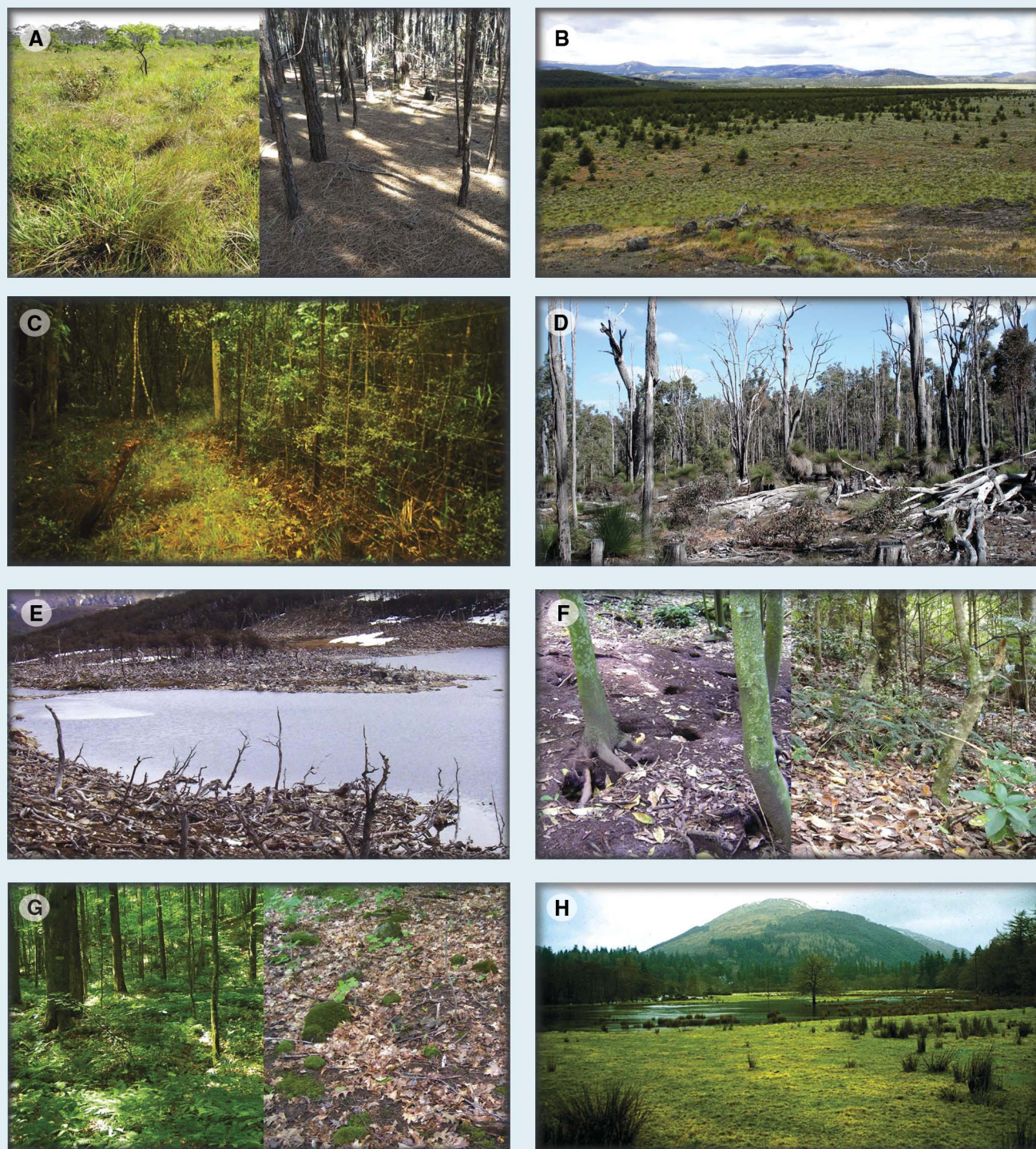
Species gain is derived from colonization and establishment of new species, processes that are increasing in frequency and intensity at global scales via cross-continental introduction, land use change, and climate warming, with demonstrated effects at the ecosystem level (Fig. 2). For plants, the development of viable populations of new species and their spread can occur rapidly as they pass through the stages of the invasion process. The impact of these species on community dynamics and ecosystem function is increasingly recognized as being correlated with the novelty of their traits relative to those of natives (8, 9). Recent studies have explored such novelty through phylogenetic relationships (9), secondary biochemistry (8), and allocation of carbon and nutrients (10). For example, a comparison in eastern Australia of leaf traits for 75 native and 90 exotic invasive plant species (11) showed that the invasives had significantly higher nitrogen and phosphorus concentrations, assimilation rates, and leaf area ratios than did the natives. As such, when invasive plants become disproportionately dominant over time, these traits can have profound effects on ecosystem properties, including local increases of nutrient stocks, rates of nutrient cycling, and primary productivity (10), although there are many exceptions (12) (Fig. 3).

Recent studies have shown that novel traits of invasive plants can undergo rapid evolution, which may either diminish (13) or increase (14) the impacts of the invaders on invaded ecosystems over time. We now also know that over time exotic plants can be subjected to increasing regulation by their enemies, which may be due to evolutionary responses of competitors or enemies that are native to the invaded range (15). As such, although community and ecosystem impacts of invasive plants may be initially strong because of their novel traits and strengthen through selection, they may not be sustained in the longer term as decomposers, herbivores, and pathogens in the

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**Fig. 2.** Invasive organisms occupying any trophic position can radically transform ecosystems when they introduce novel traits to the ecosystem. (A) Invasion of Brazilian cerrado (left) by *Pinus elliotii* and elimination of native species (right). (B) Invasion front in grassland in Patagonia (Chile) of *Pinus contorta*, which produces acidic litter that alters soil communities and impairs decomposer processes. (C) Invasive fallow deer (*Dama dama*) effects in northern New Zealand; on the left, removal by deer of palatable shrubs with high litter quality leads to domination of the ground layer by low litter quality grasses and alterations of soil food webs. (D) Dieback of Australian *Eucalyptus* forest caused by the invasive root fungal pathogen *Phytophthora cinnamoni*. (E) Extensive felling of *Nothofagus antarctica*

forest after invasion by North American beavers (*Castor canadensis*) in southern Chile. (F) Removal of forest understory and litter by nesting seabirds on offshore New Zealand islands (left) is mitigated by invasive rat (*Rattus*) species that consume seabird eggs and chicks (right). (G) Dense understory in *Acer saccharum* forest in Wisconsin (left) is greatly reduced after invasion by the burrowing earthworm *Lumbricus terrestris* (right). (H) Waterlogging and vegetation change in southern Scotland resulting from removal of burrowing flatworms by the invasive predatory New Zealand flatworm (*Aryhurdendyus triangulata*). [Photo credits: for (A), R. Callaway; (B), M. Gundale; (C), D. Wardle; (D), Dieback Working Group; (E), C. Anderson; (F, left), D. Wardle; (F, right), T. Fukami; (G), P. Ojanen; (H), B. Boag]



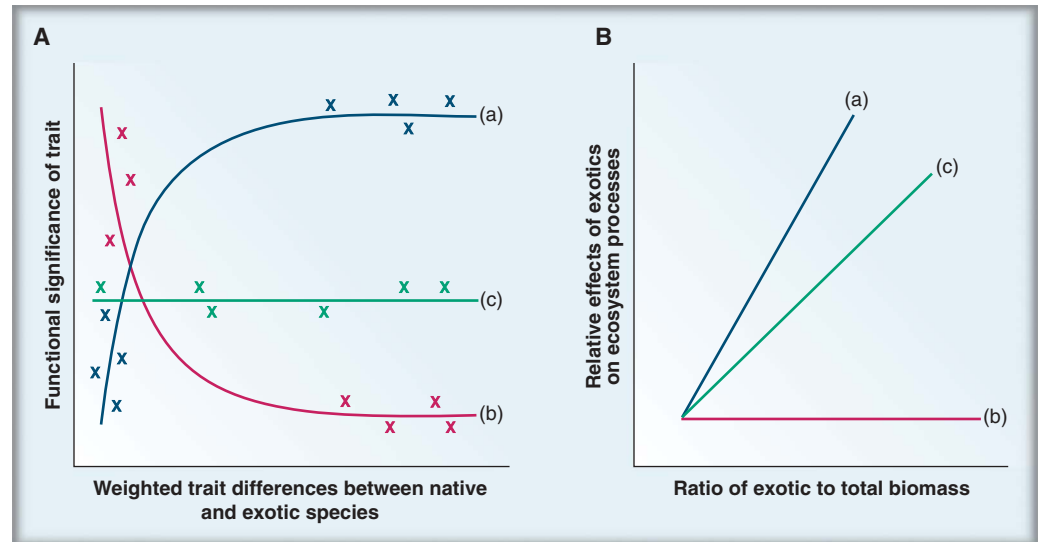
invaded range adapt (13). This dynamic process can be affected by the limited genetic variability of some invaders, which may impede evolutionary responses to biotic selection. Thus, exotic invaders may not necessarily derive longer-term benefits from aboveground and belowground enemy release or interactions with mutualistic symbionts (16).

Several recent studies have shown that gains in invasive consumers can transform ecosystems (Fig. 2), especially when host species that are poorly adapted to the invader are also ecosystem drivers (6). As such, large shifts in vegetation composition and aboveground and belowground processes may result from the invasion of functionally novel species of vertebrate and invertebrate herbivores (17), root pathogens (18), and earthworms (19) (Fig. 2). Strong ecosystem effects may result, especially when invasive consumers introduce a novel disturbance; for example, recent widespread clearance of riparian *Nothofagus* forest in southern South America by exotic beavers is thought to be the largest landscape-scale disturbance in these forests since the last ice age (20). Invasive predators also have cascading effects on ecosystem processes when they extirpate prey that itself has a key ecological role. For example, predation on seabirds by invasive foxes on the Aleutian Islands (21) and by rats on offshore New Zealand islands (22) radically transform several ecosystem properties through thwarting nutrient transfer by seabirds from the ocean to land. Despite the growing number of spectacular examples of how invasive consumers transform ecosystems (Fig. 2), relatively few general principles exist regarding what organism traits lead to new species of consumers becoming invasive (23), and even less is known about when and how these traits may transform ecosystem processes.

Global changes may promote (24) or limit (25) range expansions of plants and animals. Range-expanding organisms and their enemies probably move at different rates (26); thus, range expansion can result in disruption of both aboveground and belowground trophic interactions. Even when both prey and predator species expand their range at the same rate, the original trophic interactions may not necessarily become reunited in the new range (27), which may make range expanders behave as invaders (28). Aboveground and belowground defense traits of latitudinal range-expanding plant species can be more comparable to those of cross-continental exotic invaders than of native congeneric species (28). In climate envelope models, potential

latitudinal range expansions are estimated with climate-distribution correlations (25). Including aboveground and belowground biotic interactions in those analyses and their different response rates to global environmental changes could reveal why some species do better and others worse than when compared with model predictions. In spite of the many studies on climate warming effects on ecosystem processes, little attention has been given to ecosystem-level consequences of species gain resulting from range

expansions, functional group, genetic, or trait diversity influences ecological processes. However, their relevance for understanding species loss effects in real ecosystems continues to be debated (6, 29, 31, 32), in part because species are not lost from ecosystems at random and because small-scale effects of species loss may not be manifest at larger scales. There is growing recognition that an improved understanding of how biodiversity loss affects aboveground and belowground ecological processes requires explicit recogni-



**Fig. 3.** Role of species traits in determining how gains of exotic species within trophic levels may affect ecosystem processes. **(A)** Different relationships at the whole community level between the functional significance of traits for ecosystem processes and (standardized) biomass-weighted differences in trait values between native and exotic species, with each cross representing a different trait. (a) Situations in which those traits that differ between invasive and native species are the functionally most important, such as when N-fixing plants invade ecosystems lacking N fixers (75) or beavers invade ecosystems lacking functionally equivalent herbivores (20). (b) Cases where traits that drive ecosystem processes are different than traits that differ between invasive and native species, such as for decomposition of litter from native and invasive species on New Zealand floodplains (12). (c) A situation that is intermediate between (a) and (b). The ecosystem effect of invasive species is also determined by whether they occupy a high proportion of community biomass within their trophic level, and **(B)** shows the effects of invaders on ecosystem processes as a function of their contribution to community biomass for the scenarios for each of (a) to (c), assuming that the relationship between relative invader biomass and its effects on processes is linear; other relationships are possible. The same approach could potentially be used for understanding the ecosystem effects of species losses, by considering traits of those species lost from the community relative to those that remain, although such an approach has seldom been considered (69) and would be more challenging to implement because this also requires historical knowledge of the proportion of community biomass that was previously occupied by the lost species.

expansions or species loss resulting from range contractions.

#### Species Loss: Extinction and Range Contraction

Mechanistic understanding of how plant species gains affect community and ecosystem properties at local scales is accelerating, but knowledge of the effects of plant species losses at comparable scales remains more limited. Most work on impacts of species loss has been in the context of “biodiversity and ecosystem functioning” studies, where species richness is experimentally varied (often through random draws from a species pool) to infer how species loss in real ecosystems influences ecological processes (29, 30). Such studies have enhanced our understanding of how spe-

cies loss affects community and ecosystem properties at local scales, although there are still few direct empirical tests of this (33, 34).

One way forward for developing general principles about how plant species loss affects real ecosystems would be to take lessons from the improved understanding of invasive plant effects generated by a focus on traits associated with the invaders. This would involve determination of which traits predispose species to be lost from communities because of anthropogenic impacts and whether they are related to those traits that drive ecological processes (6, 31) (Fig. 3). For example, nitrogen enrichment often causes losses of slow-growing plant species with traits associated with poor resource quality that



impair decomposer biota, in part because they provide litter of poorer quality. Recent manipulation experiments (35, 36) show that species disadvantaged by nitrogen enrichment have different interactions with the belowground subsystem than those that benefit. As another example, effects of nonrandom removal of particular forest tree species through selective harvesting on aboveground and belowground properties depend on the characteristics of species that are lost (37). Such examples provide models for exploring ecosystem impacts of other anthropogenic species-loss scenarios. If species with particular traits that drive ecosystem processes are disproportionately (as opposed to randomly) lost from communities, then consequences for ecosystem functioning may be even greater than many biodiversity-ecosystem function experiments would predict.

The link between species traits and extinction susceptibility has attracted some attention for vertebrates. Mammals with slow growth rate, large body size, and high trophic position are disproportionately susceptible to both local and global extinction (38), and these traits may greatly affect ecosystem processes. Losses of mega-herbivore species from major land masses worldwide have caused major switches in vegetation, for example, from productive fertile steppe to infertile tundra in northern Russia (39). Further, reintroduction of locally extinct bison into tall-grass prairies in North America has revealed the dramatic effects of their historical loss on soil biogeochemical processes (40). Losses of cougars and wolves in much of North America may have caused overabundance of cervid prey, with cascading effects on vegetation, watershed hydrology (41), and soil fertility (42). Further, loss of seed-dispersing mammal species to poaching in national parks of Thailand has impaired tree seed dispersal and seedling demographics, with likely ecosystem-level impacts (43). Conversely, there are fewer examples of human-induced losses of small-bodied species affecting ecosystem processes (44), but it is unclear whether such species are less important or whether the effect of their loss is more likely to go unnoticed (6).

Losses of biodiversity also occur at sub-specific or genetic levels, but the ecological consequences of these have been infrequently studied and mostly for plants. Whereas plant species gains can result in establishment of large populations with limited genetic variation, plant species losses can be preceded by progressive reduction of genetic variation, with considerable implications for associated aboveground and belowground biota and ecosystem functioning (45). In a recent meta-analysis, plant genetic variation was shown to have greater aboveground than belowground effects, with larger impacts on consumer invertebrates of lower trophic status (46). In partial agreement with this, genotypic variation in tall goldenrod (*Solidago altissima*) was found to influence the diversity and abundance of leaf-feeding arthropods and their predators, whereas effects on litter-feeding arthropods across multi-

ple trophic levels were minimal (47). There is also emerging evidence that plant genetic variation may have ecosystem-level consequences by affecting carbon and nitrogen cycling and resource quality for litter decomposers and foliar herbivores (48). Advances in understanding consequences of lost genetic variation will require approaches that operate at the interface of community genetics, biotic interactions, and ecosystem processes.

### Understanding Consequences Within and Among Ecosystems

Most studies have considered the ecosystem impacts of species gains and losses in isolation from each other, despite both processes frequently occurring simultaneously within communities (49) and not necessarily independently. For example, invasive predators may drive ecosystem processes through causing the local extinction of their prey (21, 22). Further, loss of native plant species potentially contributes to greater success of invasive species, although the importance of this in natural ecosystems and the underlying mechanisms involved remain far from resolved (50). Whenever species gains and losses occur in the same community, we have limited knowledge about what the net consequences are for community and ecosystem processes. This is because we have scant understanding of the temporal dynamics of species loss relative to species gain in the community (49) and of whether those species that are lost from communities have comparable functional characteristics to those that are gained (6). Understanding the impact of global change on terrestrial ecosystem processes will require explicit focus on the trait spectra of those species that are both lost and gained, as well as their interactions with other trophic level biota, and how any net shift in these properties over time may impact on ecosystem functioning.

In addition to species gains and losses occurring simultaneously, there is mounting evidence that the magnitude of effects of both processes on ecosystem functioning can vary depending on environmental conditions. For example, effects of plant diversity on primary productivity depend on soil fertility (51), as does the impact of mycorrhizal fungal diversity on host plant growth (52). Likewise, removal experiments in boreal forests reveal that the impacts on belowground processes of the loss of understory species depend on soil fertility and ecosystem productivity (53). Further, the effects of the exclusion or invasion of large herbivores on ecosystem properties can vary depending on topography, geologic substrate, soil fertility, and climate (54, 55). Similarly, impacts of shrub invasion, which is occurring in arid and semi-arid grasslands worldwide, on primary production and soil biogeochemistry vary with climatic conditions (56). Such context dependency calls for an improved understanding of how the balance between species gain and loss may be affected by environmental conditions and how the result-

ing net shifts in trait spectra may determine ecosystem properties.

Although few studies have explicitly addressed how climate change may accelerate modification of terrestrial ecosystems through species interchange, the widespread climate-mediated expansion of many organisms (24, 57) has implications for local-scale ecosystem properties and potentially the Earth system. For example, pan-arctic shrub encroachment is predicted to retard decomposition rates because of production of poorer quality leaf and woody litter than that of the species that are replaced (58). This may dampen atmospheric CO<sub>2</sub> inputs caused by warming effects on organic matter decomposition and carbon loss from arctic soils (59). Also, climate-mediated expansion of the mountain pine beetle (*Dendroctonus ponderosae*) may convert forests from being net carbon sinks to net sources, thereby reducing their capacity to offset anthropogenic CO<sub>2</sub> emissions (60). Further, climate-mediated range expansion of large herbivores (61) can result in shifts in vegetation composition and net ecosystem carbon exchange (6) and modulate climate change effects on vegetation, for instance, by constraining woody plant expansion in response to warming (62). Research is needed to better understand the potential for species range expansion to modulate ecosystem functioning under global change and to unravel the relative importance of factors that determine the scale of these impacts. In addition, comparisons of the relative importance of the characteristics of the shifting species and the habitats into which they expand, as well as the environmental changes that might cause range shifts, will allow us to better target the drivers of range shifts.

Species gains and losses also have important implications for the future management and restoration of ecosystems. Restoration of ecosystems transformed by invaders not only requires an understanding of how the ecosystem functioning has been modified but also how reversible these effects are if the invader is removed (63, 64). This requires consideration of whether the loss of associated biota, including soil organisms, may limit subsequent restoration of native communities and their capacity to deliver ecosystem functions. Restoration is also required to counteract species loss, for example, of plant species in grasslands subject to nutrient enrichment. Restoration of plant diversity in such situations is primarily constrained by high soil fertility, seed limitation of later-successional target species (65), and degraded soil communities (66). As a consequence, more integrated aboveground-belowground interventions are needed to facilitate restoration of plant diversity, including situations where some species have been lost while others have invaded (63), and to potentially enhance delivery of ecosystem services such as carbon sequestration (67).

### Conclusions and a Way Forward

The functioning of many ecosystems worldwide has been transformed, sometimes spectacularly,

by the losses of some species and the gains of others. However, our ability to generalize about or predict when and how interchange of biota alters ecosystem properties is incomplete. There have been recent advances in understanding how invasive plant species alter aboveground and belowground properties. However, we need to better understand why most alien species do not become invasive and whether the most invasive alien species are also those with novel attributes that transform ecosystems (68). Despite many examples of major alterations of ecosystems by invasive consumers (Fig. 2), we have yet to move from a collection of impressive examples to the development of general principles about how, when, and where alien consumer species may exert such effects. Our ability to generalize about the impacts of species losses in real ecosystems requires more experimental studies that include realistic and nonrandom species loss scenarios, as well as the environmental factors that drive species losses. Crucially, although species gains and losses occur simultaneously in many communities, we still have a dearth of knowledge about the net consequences of the two processes occurring in tandem for terrestrial ecosystems functioning. As such, the advancement of this topic will require studies that explicitly consider both species invasion and extinction, as well as their interactions, environmental drivers, and temporal dynamics.

To advance understanding of ecosystem impacts of species gains and losses either separately or in combination, we highlight three areas that deserve attention. First, a trait-based framework analogous to current “effect trait” and “response trait” frameworks (26, 69) has much to offer. This could focus on whether those traits that predispose species to alter ecosystems are also associated with invasiveness or extinction (Fig. 3). Second, greater attention is needed on whether effects of species gains or losses on ecosystem properties are mostly a consequence of their relative abundance or biomass (70) or whether gains of subordinate species (71) or losses of rare species (34) are also important. Third, despite growing evidence that effects of species gains and losses on ecosystem properties vary greatly among ecosystems, we lack understanding of what underpins this context dependency. Experimental and observational studies that investigate the impacts of species gains and losses across contrasting ecosystems and environmental gradients could assist our knowledge about how interchange of species and abiotic factors interact to drive ecosystem properties. Further, because there are conceptual similarities between how gains and losses affect ecosystems (Fig. 1), approaches used to study one can generate new insights into the other.

Improved understanding of the ecosystem consequences of interchange of species will also assist our ability to evaluate the future consequences of global environmental change. First, predictions of range shift because of climate change, such as those obtained by climate envelope models, are based largely on abiotic

factors. However, biotic interactions can govern species abundance after their range expansion (28) and potentially the climate envelope of species invading new regions (72). Explicit recognition of the need to include biotic interactions in novel climate envelope models is growing, and this will help more accurate prediction of species distributions and ecosystem effects under global change scenarios (73). Second, large-scale transformations of ecosystems after species gains or losses may have important, although largely unrealized, consequences for ecosystem carbon fluxes (74) that feed back to the Earth climate system (6). Gains and losses of species from ecosystems are both a consequence and driver of global change, and understanding the net consequences of this interchange for ecosystem functioning is key to determining the response of the Earth system to current and future human activities.

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# Microcredit in Theory and Practice: Using Randomized Credit Scoring for Impact Evaluation

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Microcredit institutions spend billions of dollars fighting poverty by making small loans primarily to female entrepreneurs. Proponents argue that microcredit mitigates market failures, spurs micro-enterprise growth, and boosts borrowers' well-being. We tested these hypotheses with the use of an innovative, replicable experimental design that randomly assigned individual liability microloans (of \$225 on average) to 1601 individuals in the Philippines through credit scoring. After 11 to 22 months, we found evidence consistent with unmet demand at the current price (a roughly 60% annualized interest rate): Net borrowing increased in the treatment group relative to controls. However, the number of business activities and employees in the treatment group decreased relative to controls, and subjective well-being declined slightly. We also found little evidence that treatment effects were more pronounced for women. However, we did find that microloans increase ability to cope with risk, strengthen community ties, and increase access to informal credit. Thus, microcredit here may work, but through channels different from those often hypothesized by its proponents.

Microcredit—broadly speaking, the provision of small loans (typically \$100 to \$500 U.S.) to very small businesses, typically self-run enterprises with few if any employees—is an increasingly common weapon in the fight to reduce poverty and promote economic growth (1). The motivation for the continued expansion of microcredit, or at least for the continued flow of subsidies to both nonprofit and for-profit lenders, is the presumption that expanding credit access is a relatively efficient way to fight poverty and promote growth. Yet despite strong claims about the effects of microcredit on borrowers and their businesses (e.g., the 2006 Nobel Peace Prize to Muhammad Yunus and the Grameen Bank), there is relatively little rigorous evidence about these programs (2).

In practice, the policy discussion about micro-lending typically emphasizes the upside, arguing that microlending mitigates market failures (by making access to credit possible for entrepreneurs previously unable to get credit), empowers women (e.g., improves their decision-making power by giving them more financial independence), and spurs enterprise growth and improves subjective well-being (e.g., increase in life satisfaction, self-esteem, and optimism; decrease in level of stress).

In theory, expanding credit access may not have positive effects on borrowers and could even

have negative effects. Financial institutions may disrupt relatively efficient “informal” (community- or family-based) mechanisms (3). The often high cost of microcredit, from 10% annualized interest rates to 100%, means that even higher returns to capital are required for microcredit to produce improvements in business income, and thus in household income and consumption. Finally, some argue that psychological biases may induce some to “overborrow” and do themselves more harm than good (4).

“Traditional” microlenders target women who operate small-scale businesses and use group lending mechanisms (5). But as microlending has expanded and evolved into its “second generation,” it often ends up looking more like traditional retail or small-business lending: For-profit lenders extend individual liability credit in increasingly urban and competitive settings. For example, recent estimates suggest that about one-half of microfinance institutions are individual liability lenders, and about one-quarter are for-profits or cooperatives (6–9).

We have conducted a randomized evaluation of second-generation microcredit by working with First Macro Bank (FMB) to implement a novel, replicable experimental design that uses credit scoring to randomly assign individual liability loans as a source of exogenous variation in credit access (10–12). FMB is a for-profit lender that makes small, 3-month loans at 60% annualized interest rates to micro-entrepreneurs in the outskirts of Manila and receives technical assistance from a U.S. Agency for International Development (USAID) contractor (13). Nonrandomized empirical evaluations of microcredit impacts are typically complicated by classic endogeneity problems: Client self-selection and lender strategy

likely produce correlations among credit access, ultimate outcomes, and critical unobserved inputs (e.g., client opportunity sets, preferences, and aptitude) that confound attempts to make causal inferences of microcredit impacts (14).

We worked with the lender to build a quantitative model that distinguishes creditworthy or uncreditworthy applicants from marginal ones. Marginal applicants then get approved for a loan according to some preassigned probability. This method provides lenders with a way to take systematic, controlled risks when refining underwriting strategies. It also provides researchers and policymakers with a source of exogenous variation in access to credit that may be used, in conjunction with follow-up data (e.g., on business and household outcomes), to help identify the impacts of microcredit from a change in the screening criteria of existing lenders on marginal applicants. Note that impacts may differ for infra-marginal applicants; we discuss this and other external validity issues below. Nonetheless, our methodology is transferable to many different types of lenders and settings.

The ability to transfer an evaluation method to a range of contexts is particularly important given the unsettled state of evidence on micro-finance impacts. Prior studies have used various methodologies to address endogeneity problems and have found varied impacts or lack thereof (15–23). Is the variation in estimated impacts across studies due to methodology, to true underlying heterogeneity in borrower characteristics and market conditions, or to both? In particular, we draw the reader's attention to (2), which provides a summary of results and methodological issues of several nonexperimental impact evaluations to date. Applying similar experimental methodological approaches across different settings will help us to address these issues and paint a more complete picture for theory testing and policy evaluation.

**Setting.** Our cooperating lender, FMB, has operated as a rural bank in the metro Manila region of the Philippines since 1960. Filipino “microlenders” include both for-profit and nonprofit lenders offering small loans to micro-entrepreneurs (average \$220) that are short-term (less than 1 year), uncollateralized, and on fixed schedules of equal periodic repayments.

Most Filipino microlenders operate on a small scale relative to microfinance institutions (MFIs) in the rest of Asia (24), and our lender is no exception. FMB maintained a portfolio of about 1400 individual and 2000 group borrowers throughout the course of the study. This portfolio represents a small fraction of its overall lending, which also includes larger business and consumer loans as well as home mortgages.

Microloan borrowers typically lack the credit history and/or collateralizable wealth needed to borrow from traditional institutional sources such as commercial banks. This holds for our sample—which is only marginally creditworthy by FMB's

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standards—even though our subjects have average income and education levels. Table 1 provides some demographics on our sample frame relative to the rest of Manila and the Philippines.

Casual observation suggests that many micro-entrepreneurs in our study population face binding credit constraints. Credit bureau coverage of micro-entrepreneurs in the Philippines is quite thin, so building a credit history is difficult for many business owners and consumers. Informal credit markets are robust, and serial borrowing from moneylenders charging 20% or more per month is common (more than 30% of our sample reported borrowing from moneylenders during the past year). Trade credit (i.e., credit from suppliers) is quite uncommon.

The loan terms granted in this experiment were the lender's standard ones for first-time borrowers. Loan sizes ranged from 5000 to 25,000 pesos, which is substantial relative to borrower income. For example, the median loan size made under this experiment, 10,000 pesos (\$220), was 37% of the median borrower's net monthly income. Loan maturity was 13 weeks, with weekly repayments. The monthly interest rate was 2.5%, charged over the declining balance. Several upfront fees combined with the interest rate to produce an effective annual interest rate greater than 60% (25).

The lender conducted underwriting and transactions in its branch network. At the onset of this study, FMB changed its risk assessment process from one based on weekly credit committee meetings to one that used computerized credit scoring.

Delinquency and default rates were substantial. One-third of the loans in our sample were paid late at some point, and 7.4% were charged off.

**Methods.** We drew our sample frame from the universe of several thousand applicants who applied at eight of the lender's nine branches between 10 February 2006 and 16 November 2007 (26). The branches were located in the provinces of Rizal, Cavite, and the National Capital Region. The lender maintained normal marketing procedures by having account officers canvass public markets and hold group information sessions for prospective clients, and these sessions did not include explicit discussion of the credit-scoring procedures.

Our research design uses credit-scoring software to randomize the approval decision for marginally creditworthy applicants (although "marginal" in this case actually encompassed 74% of the sample frame), and then uses data from household/business follow-up surveys to measure impacts on credit access and on several classes of more ultimate outcomes of interest.

The survey data are collected by a firm, hired by the researchers, that has no ties to the lender.

Table 1 provides some summary statistics, from ex ante application data, on our sample frame of 1601 marginally creditworthy applicants, nearly all (1583) of whom were first-time applicants to the lender. Our sample was largely female, with a typical household size and with educational attainment and household income in line with averages for Metro Manila. The most common business was a sari-sari (small grocery/convenience) store. Other common businesses were food vending and various services (e.g., auto and tire repair, water supply, tailoring, and barbers and salons).

The lender identified marginally creditworthy applicants by means of a proprietary credit-scoring algorithm developed collaboratively between the lender and the researchers, based on business capacity, personal financial resources, outside financial resources, personal and business stability, and demographic characteristics (27). Credit bureau coverage of our study population is very thin, and our lender does not use credit bureau information as an input into its scoring. Scores ranged from 0 to 100, with applicants scoring below 31 rejected automatically and applicants scoring above 59 approved automatically. Our 1601 marginally creditworthy applicants fell into two randomization "windows": low (scores 31 to 45, with

**Table 1.** Demographics. Sample frame data taken from lender's application data unless otherwise noted. Per capita figures for Manila and the Philippines assume an average household size of 5.0 people. Source: [www.census.gov.ph/data/quickstat/index.html](http://www.census.gov.ph/data/quickstat/index.html).

|                                                      | Our sample frame |        |                                        |        |                                        |        | Metro Manila |        | Philippines |        |
|------------------------------------------------------|------------------|--------|----------------------------------------|--------|----------------------------------------|--------|--------------|--------|-------------|--------|
|                                                      | All              |        | Applicants with 60% chance of approval |        | Applicants with 85% chance of approval |        | Mean         | Median | Mean        | Median |
|                                                      | Mean             | Median | Mean                                   | Median | Mean                                   | Median |              |        |             |        |
| Applicant is female                                  | 85%              | —      | 86%                                    | —      | 85%                                    | —      |              |        |             |        |
| Applicant is married                                 | 78%              | —      | 53%                                    | —      | 82%                                    | —      |              |        |             |        |
| Age of applicant                                     | 42.1             | 42.0   | 41.8                                   | 42.0   | 42.1                                   | 42.0   |              |        |             |        |
| Education level of applicant*                        |                  |        |                                        |        |                                        |        |              |        |             |        |
| Elementary                                           | 11%              | —      | 19%                                    | —      | 10%                                    | —      | 12%          |        | 33%         |        |
| High school                                          | 44%              | —      | 49%                                    | —      | 43%                                    | —      | 42%          |        | 37%         |        |
| Postsecondary or college                             | 45%              | —      | 32%                                    | —      | 47%                                    | —      | 47%          |        | 31%         |        |
| Household size                                       | 5.1              | 5.0    | 5.0                                    | 5.0    | 5.1                                    | 5.0    | 5.0          |        | 5.0         |        |
| Number of dependents                                 | 2.28             | 2      | 2.29                                   | 2      | 2.28                                   | 2      |              |        |             |        |
| Applicant owns a sari-sari (corner) store            | 49%              | —      | 55%                                    | —      | 48%                                    | —      |              |        |             |        |
| Monthly household income (Filipino pesos)†           | 24,920           | 17,245 | 19,524                                 | 14,150 | 25,826                                 | 17,800 | 25,917       | 18,333 | 14,417      | 9250   |
| Monthly household income per capita (Filipino pesos) | 5301             | 3540   | 4193                                   | 3191   | 5488                                   | 3569   | 5183         | 3667   | 2883        | 1850   |
| Number of businesses owned by household              | 1.15             | 1      | 1.20                                   | 1      | 1.14                                   | 1      |              |        |             |        |
| Applicant's business has employees                   | 25%              | —      | 17%                                    | —      | 26%                                    | —      |              |        |             |        |
| Number of loans with First Macro Bank during study   | 2.0              | 2      | 2.3                                    | 2      | 2.0                                    | 2      |              |        |             |        |

\*Education data on sample frame are from the follow-up survey, where 97% of the sample frame is aged 20 to 59. Education data on Manila and the Philippines, restricted to Filipinos aged 20 to 59, are from [www.census.gov.ph/data/sectordata/2003/fl03tabA.htm](http://www.census.gov.ph/data/sectordata/2003/fl03tabA.htm).

†Monthly household income data on sample frame taken from the following questions from the follow-up survey: "How much was the total income (including remittances) earned by your household in the past month (gross calculation before expenses)?" less the sum total of "How much did each household business spend on each of the following categories of business expenses during the past month: [inventory, utility bills, wages and salaries for helpers, rent for machinery and equipment, rent for building and land, taxes, maintenance and general repairs, business-related transportation, and other expenses]?" Monthly household income data on Manila and the Philippines are from [www.census.gov.ph/data/sectordata/2006/fies0607r.htm](http://www.census.gov.ph/data/sectordata/2006/fies0607r.htm), where, according to the Family Income and Expenditures Survey, "total family income includes primary income and receipts from other sources received by all family members ... and net receipts derived from the operation of family-operated enterprises/activities."



60% probability of approval) and high (scores 46 to 59, with 85% probability of approval). The randomization was opaque to loan officers, their direct supervisors, and applicants, in the sense that none of these parties saw actual credit scores, knew the details of the algorithm, or knew there was a random component to application decisions. In total, 1272 applicants were assigned to the treatment (loan approval) group, leaving 329 in the control (loan rejection) group.

The motivation for experimenting with credit access on a pool of marginal applicants is two-fold. First, it focuses on those who are targeted by initiatives to expand access to credit, because those with higher credit scores are likely to have easier access to credit in general. Second, (randomly) approving some marginally creditworthy applicants generates data points on the lender's profitability frontier (by taking controlled risks). This feeds into revisions to the credit-scoring model and helps improve lender profits.

Table S1 provides some confirmation of two key conditions needed for our study design to produce exogenous variation in access to credit. First, the randomization was implemented properly: Pretreatment characteristics did not predict assignment to treatment in the full sample, in the sample that completed the follow-up survey, for females, or for men. Second, treatment assignment did not influence follow-up survey completion, which was not predicted by assignment to treatment, nor by interaction of assignment to treatment and baseline covariates (28).

Our sample frame and treatment assignments were created in the flow of the lender's three-step credit-scoring process. This process is replicable because it is relatively easy to administer operationally. Moreover, it can be augmented to introduce random assignment into other elements of loan contracting besides the approve/reject decision: pricing, loan amount, maturity, etc.

First, loan officers screened potential applicants on FMB's "basic four requirements" (18 to 60 years old, in business for at least 1 year, in residence for at least 1 year if homeowner or at least 3 years if renter, and daily income of at least 750 pesos); 2158 applicants passed this screen. Second, loan officers entered household and business information on these 2158 applicants into the credit-scoring software, and the software then rendered its application disposition within seconds. Of this group, 391 applications received scores in the automatic approval range, and 166 applications received scores in the automatic rejection range. The remaining 1601 applicants had scores in one of the two randomization windows (approve with 60% or 85% probability), and this group constituted our sample frame. Of these 1601 marginal applicants, 1272 were assigned "approve" and 329 applicants were assigned "reject" by the software, which simply instructed loan officers to approve or reject; that is, it did not display the application score or make any mention of the randomization. Neither loan officers, branch managers, nor applicants were informed about the credit-scoring algorithm or its random component.

The credit-scoring software's decision was contingent on complete verification of the application information, so the third step involved any additional due diligence deemed necessary by the loan officer or his supervisor. Verification steps included visits to the applicant's home and/or business, meeting with neighborhood officials, and checking references (e.g., from other lenders). If loan officers found discrepancies, they updated the information in the credit-scoring software, and in some cases the software changed its decision from approve to reject. In other cases, applicants decided not to go forward with completing the application, or completed the application successfully but did not avail the loan.

In all, there were 351 applications assigned out of the 1272 assigned to treatment that did not ultimately result in a loan. Conversely, there were five applications assigned to the control (rejected) group that did receive a loan (presumably because of loan officer noncompliance or clerical errors).

In all cases, we used the original treatment assignment from step 2 to estimate treatment effects; that is, we used the random assignment (loan approval or rejection) to estimate intention-to-treat effects: the treatment effect on all randomly assigned to receive credit, irrespective of whether the bank complied with the random assignment. One alternative, the treatment on the treated (TOT), would instead scale the intention-to-treat estimate up by the reciprocal of the compliance rate, and then present the estimated treatment effect on those who do ultimately receive the treatment.

**Table 2.** Microcredit in theory: Intention-to-treat effects of credit access on widely hypothesized outcomes. For the full sample and for females, data are OLS results for the independent variable "assigned a loan"; Huber-White SEs and control group means for the dependent variable listed in each row are also shown. The incremental effect on males is shown as an estimate for the interaction between "assigned a loan" and "male." Variation in sample sizes

is due to survey question nonresponse. The summary index is in standard deviation units of the average outcome of its components. All estimates control for probability of assignment to treatment and for timing of treatment assignment and survey measurement. Borrowing measures do not count the 1% of loans that are too large (>50,000 pesos) to be plausibly affected by the treatment.

|                                                                                                                        | Full sample |       |                    | Females    |       |                    | Incremental effect on males |       |                    |
|------------------------------------------------------------------------------------------------------------------------|-------------|-------|--------------------|------------|-------|--------------------|-----------------------------|-------|--------------------|
|                                                                                                                        | OLS result  | SE    | Control group mean | OLS result | SE    | Control group mean | Estimated interaction       | SE    | Control group mean |
| Borrowing                                                                                                              |             |       |                    |            |       |                    |                             |       |                    |
| Number of loans from financial institutions in month before survey                                                     | 0.094**     | 0.045 | 0.359              | 0.080      | 0.051 | 0.385              | 0.039                       | 0.095 | 0.244              |
| Number of loans from friends, family, or moneylenders in month before survey                                           | -0.011      | 0.042 | 0.286              | -0.011     | 0.045 | 0.279              | 0.010                       | 0.104 | 0.317              |
| Business size                                                                                                          |             |       |                    |            |       |                    |                             |       |                    |
| Number of businesses in household                                                                                      | -0.102*     | 0.060 | 1.378              | -0.057     | 0.062 | 1.354              | -0.265                      | 0.181 | 1.488              |
| Number of paid employees (not including in-kind contributions) in all household businesses                             | -0.273**    | 0.123 | 0.878              | -0.214     | 0.130 | 0.801              | -0.272                      | 0.417 | 1.220              |
| Subjective well-being                                                                                                  |             |       |                    |            |       |                    |                             |       |                    |
| Life satisfaction (scale: 1–4, 1 = not at all, 4 = very)                                                               | 0.016       | 0.063 | 2.818              | -0.024     | 0.067 | 2.855              | 0.209                       | 0.168 | 2.659              |
| Job stress (scale: -12 to 0: 0 = no stress, -12 = always stressed)                                                     | -0.190      | 0.227 | -6.725             | 0.033      | 0.254 | -6.912             | -1.189**                    | 0.513 | -5.925             |
| Summary index of above outcomes, optimism, calmness, worry, job satisfaction, decision power, and socioeconomic status | -0.053*     | 0.030 | 0.000              | -0.043     | 0.032 | -0.014             | -0.042                      | 0.082 | 0.064              |
|                                                                                                                        |             |       | N = 1062–1113      |            |       |                    | N(male) = 160–165           |       |                    |

\*P < 0.10, \*\*P < 0.05, \*\*\*P < 0.01.

We report the intention-to-treat estimate because it more closely maps into the policy parameter of interest: the effect of a credit expansion where the final disposition of the application rests on some discretion by the borrower and/or the loan officer.

After the experiment, we hired researchers from a local university to survey all 1601 applicants in the treatment and control groups. The stated purpose of the survey was to collect information on the financial condition and well-being of micro-entrepreneurs and their households. As detailed below, the surveyors asked questions on business condition, household resources, demographics, assets, household member occupation, consumption, subjective well-being, and political and community participation. Neither the survey firm nor the respondents were informed about the experiment or any association with the lender, so as to avoid potential response bias in the treatment group relative to the control group.

Surveyors completed 1113 follow-up surveys, for a 70% response rate (of the 30% attrition, 81% were not found and 19% refused to be surveyed). Table S1 shows that survey completion was not significantly correlated with treatment assignment, nor with interaction of treatment assignment and baseline covariates in a Wald test.

Ninety-nine percent of the surveys were conducted within 11 to 22 months of the date on which the applicant entered the experiment by applying for a loan and being placed in the pool of marginally creditworthy applicants. The mean number of days between treatment and follow-up was  $411 \pm 76$  (SD).

We then used survey data to measure outcomes  $Y$  for estimating intention-to-treat effects with the ordinary least-squares (OLS) specification:

$$Y_i^k = \alpha + \beta^k \text{Assignment}_i + \delta \text{Risk}_i + \phi \text{APP\_WHEN}_i + \gamma \text{SURVEY\_WHEN}_i + \varepsilon_i \quad (1)$$

where  $k$  indexes different outcomes (e.g., number of formal sector loans in the month before the survey; life satisfaction) for applicant  $i$  (or  $i$ 's household).  $\text{Assignment}_i = 1$  if the individual was assigned to treatment (regardless of whether they actually received a loan).  $\text{Risk}_i$  captures the applicant's credit-score window (low or high); the probability of assignment to treatment was conditional on this (set to either 0.60 or 0.85, depending on their credit score), and thus it is necessary to include this as a control variable in all specifications.  $\text{APP\_WHEN}$  is a vector of indicator variables for the month and year in which the ap-

plicant entered the experiment;  $\text{SURVEY\_WHEN}$  is a vector of indicator variables for the month and year in which the survey was completed. These variables control flexibly for the possibility that the lag between application and survey is correlated with both treatment status and outcomes.

**Results.** The first part of our evaluation focuses on how microcredit is supposed to work in theory, by testing four key hypotheses put forth by microcredit advocates.

*H1: Microcredit mitigates market failures.* The theory of microcredit focuses on how it alleviates asymmetric information and credit rationing (29, 30). If rationing exists, then there will be excess demand for credit even at market rates (i.e., prices will not clear markets as predicted by canonical neoclassical models). We test whether there was ex ante rationing, and by implication market failure, in our setting by using different types of borrowing as dependent variables in our estimating equation.

Table 2 presents the key results, focusing on "counting" outcomes and a 1-month recall period to minimize noise. We find that FMB's microcredit expansion, at market rates, did significantly increase borrowing from financial institutions. This result is consistent with H1. The point estimate

**Table 3.** Microcredit in practice: Intention-to-treat effects on household risk management. For the full sample and for females, impacts on trust outcomes are estimated using ordered probit, and other data are OLS results for the independent variable "assigned a loan"; Huber-White SEs and control group means for the dependent variable listed in each row are

also shown. The incremental effect on males is shown as an estimate for the interaction between "assigned a loan" and "male." Variation in sample sizes is due to survey question nonresponse. All estimates control for probability of assignment to treatment and for timing of treatment assignment and survey measurement.

|                                                                                                             | Full sample                  |       |                        | Females                      |       |                    | Incremental effect on males        |       |                    |
|-------------------------------------------------------------------------------------------------------------|------------------------------|-------|------------------------|------------------------------|-------|--------------------|------------------------------------|-------|--------------------|
|                                                                                                             | OLS or ordered probit result | SE    | Control group mean     | OLS or ordered probit result | SE    | Control group mean | Estimated interaction              | SE    | Control group mean |
| Financial instruments                                                                                       |                              |       |                        |                              |       |                    |                                    |       |                    |
| Any health insurance                                                                                        | −0.035                       | 0.038 | 0.658                  | −0.018                       | 0.043 | 0.646              | −0.101                             | 0.094 | 0.707              |
| Any other type of insurance                                                                                 | −0.079**                     | 0.039 | 0.486                  | −0.066                       | 0.043 | 0.475              | −0.072                             | 0.101 | 0.537              |
| Any savings in household                                                                                    | 0.002                        | 0.039 | 0.591                  | −0.009                       | 0.043 | 0.594              | 0.063                              | 0.099 | 0.575              |
| Family/community networks                                                                                   |                              |       |                        |                              |       |                    |                                    |       |                    |
| Trust that you would not be taken advantage of (1 = people would take advantage, 10 = people would be fair) | −0.060                       | 0.082 | 7.685                  | −0.087                       | 0.092 | 7.725              | 0.150                              | 0.192 | 7.512              |
| Trust in your neighborhood (−4 = no trust, −1 = complete trust)                                             | 0.209**                      | 0.090 | −2.215                 | 0.203**                      | 0.101 | −2.219             | 0.064                              | 0.196 | −2.195             |
| Trust in people you know personally (−4 = no trust, −1 = complete trust)                                    | 0.036                        | 0.093 | −1.895                 | 0.001                        | 0.102 | −1.882             | 0.215                              | 0.237 | −1.951             |
| Trust in your business associates (−4 = no trust, −1 = complete trust)                                      | 0.101                        | 0.089 | −2.184                 | 0.080                        | 0.101 | −2.175             | 0.117                              | 0.186 | −2.225             |
| Could get financial assistance from family or friends in an emergency                                       | 0.010                        | 0.027 | 0.883                  | −0.003                       | 0.030 | 0.888              | 0.080                              | 0.068 | 0.861              |
| Could get 10,000 pesos' worth of financial assistance from family or friends in an emergency                | 0.102***                     | 0.040 | 0.370                  | 0.091**                      | 0.044 | 0.379              | 0.062                              | 0.102 | 0.333              |
| Could get unlimited financial assistance from family or friends in an emergency                             | 0.090**                      | 0.035 | 0.254                  | 0.074*                       | 0.039 | 0.267              | 0.091                              | 0.087 | 0.194              |
|                                                                                                             |                              |       | $N = 995\text{--}1113$ |                              |       |                    | $N(\text{male}) = 151\text{--}165$ |       |                    |

\* $P < 0.10$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .



implies an economically large effect: a 9.4 percentage point (26%) increase in the proportion of individuals with formal loans. We find no effect on “informal” borrowing: loans from friends, family, and moneylenders (i.e., no crowding out of other debt). Results for other measures of borrowing, and for a 1-year recall period, paint a similar picture (one interesting exception is that we do find a significant treatment effect reducing the likelihood of any informal borrowing over the past year).

The size of the treatment effects may be understated here for two reasons: (i) underreporting of borrowing [e.g., more than half of borrowers known, from FMB’s data, to have a loan outstanding from FMB in the month before the survey do not report any borrowing in the survey; see (31) for a similar result from a different setting]; and (ii) the timing of the recall period, which may miss earlier (short-run) effects, particularly for borrowers who needed/took only one loan, because the follow-up survey took place 11 to 22 months after treatment assignment, and the initial loan term was only 4 months.

**H2: Microcredit spurs business growth.** We focus here on two key proxies for the scale of business activity undertaken by our subjects’ households. Both are relatively easy to measure precisely. One is the number of businesses the household is operating; the other is the number of paid employees. The results suggest that expanded access to credit shrinks business scale if anything: Our treatment group business owners operate 0.1 fewer business (7% less than the control group mean;  $P = 0.09$ ,  $t$  test) and have 0.27 (31%) fewer paid employees. These results do not support H2. Results for three other proxies for business size and success—total profits, gross sales, and inventory—are noisy and hence do not sharpen inference with respect to H2 (table S4).

**H3: Microcredit improves subjective well-being.** Microcredit proponents argue that having additional choices makes borrowers feel more capable, optimistic, and happy. We use several measures of subjective well-being to test these hypotheses. Table 2 shows that we find a precisely estimated null effect on life satisfaction (32). Nor do we find any change in stress in the full sample, although, as in Fernald *et al.* (33), we do find a significant (and significantly greater) increase in stress for male borrowers. Combining these and several other standard measures into a summary index of subjective well-being (12), we find that expanded credit access produces a small (1/20 of a standard deviation;  $P = 0.08$ ,  $t$  test) decrease in subjective well-being. In short, we find no support for H3. One important question, beyond the scope of the present study, is whether (subjective well-being) benefits are specific to the lending mechanism; for example, perhaps group lending produces some benefits that individual lending does not (or vice versa).

**H4: There are disproportionate benefits from targeting women.** We test this, for each outcome discussed above, by interacting the treatment as-

signment and gender. We find little evidence of significantly different treatment effects for women (Table 2); the one exception is that males experience a greater increase in stress. Two important caveats are that we lack the statistical power to detect some economically meaningful differences, and that only 15% of our sample is male (raising questions about external validity).

The second part of our evaluation presents some evidence on how microcredit works in practice, by testing two additional hypotheses suggested by the literature on risk sharing (8, 34). Results appear in Table 3.

**H5: Microcredit is a substitute for insurance and precautionary saving.** We test this by looking at three common ways that households mitigate risk. We find a 3.5 percentage point reduction ( $P = 0.36$ ,  $t$  test) for the treatment effect on holding any health insurance, and a 7.9 percentage point reduction, significant at 5% ( $P = 0.043$ ,  $t$  test), in holding other types of insurance (life, home, property, fire, and car). These results suggest that microcredit allows households to conserve resources by substituting away from formal insurance to other methods of managing risk. We find no effect on the likelihood of holding savings, with the caveat that there are motives besides precautionary ones for saving. In all, we find limited support for H5, in that access to credit led individuals to lower their demand for formal insurance. This suggests that microcredit is used to buffer fluctuations in income and expenses.

**H6: Microcredit improves informal risk-sharing.** In theory, this can go either way: Microcredit may disrupt informal arrangements because outside options weaken commitments to reciprocate, or it may strengthen informal arrangements by providing participants with greater resources and liquidity. We find evidence for the latter effect (i.e., for H6). Trust in one’s neighborhood increases for the treatment group, as do two other trust measures (35). The treatment group also reports greater access to informal credit (as measured by responses to questions about how much financial assistance one could get from friends or family in an emergency).

In all, these results suggest that microcredit improves the ability of household to manage risk by giving them additional options: using credit instead of insurance (or precautionary saving), and strengthening family and community risk-sharing.

**Discussion.** We used the random assignment of loan offers via credit scoring to generate evidence on the effects of individual liability microcredit on micro-entrepreneurs in Manila. The results are surprising. Entrepreneurs who were randomly assigned microloans shrank their businesses, and their subjective well-being did not improve. The benefits we found—that microcredit access improves risk management—are on margins often deemed second-order by policymakers, practitioners, and economists. We did not find any evidence that treatment effects are more pronounced for female borrowers.

The overall picture of our results questions the wisdom of assuming that impacts are stronger for preexisting micro-entrepreneurs and women than for “consumers,” men, or aspiring micro-entrepreneurs. Money is fungible, and we find that entrepreneurs do not necessarily invest loan proceeds in growing their businesses. Perhaps micro-entrepreneurs only increase business investment if loan proceeds are tied to a more detailed business planning exercise or are followed by unusually close monitoring from the lender. In any case, limiting microcredit access to entrepreneurs may forgo opportunities to improve human capital and risk-sharing for non-micro-entrepreneurs (37).

Above all, our results highlight the importance of replicating tests of theories and interventions across different settings. Our findings add to a very muddled picture on the impacts (or lack thereof) of microcredit. At this point it remains to be seen whether different studies arrive at different estimates because of true underlying heterogeneity across settings, to different targeting [e.g., Banerjee *et al.* (11) found positive impacts on investment in new micro-enterprises but no growth of preexisting micro-enterprises], and/or to differences (and flaws) in some methodologies. One approach to solving this puzzle is to replicate research designs across settings.

Random assignment via credit scoring is a viable tool for replication, as it provides a win-win for lenders looking for an effective way to improve operations, and for other constituencies (researchers, donors, investors, and policymakers) looking for an effective way to measure impacts of expanding access to microcredit. It also provides a way to maximize statistical power given budget and/or operational constraints; the leading alternative randomized method, randomized program placement, will typically require much larger sample sizes because of clustering issues and lower take-up rates (38).

Furthermore, regarding statistical power, this study and other similar studies are hindered by low differential take-up in treatment versus control, as well as limited resources for follow-up data collection. Given the results of this study and other similar studies, we suggest that further studies would benefit from larger sample sizes, from greater focus on operational issues that could expand the differential take-up rate of treatment versus control, and perhaps from more waves of follow-up data, in order to expand the set of hypotheses that can be tested with sufficient power.

Nonetheless, random assignment via credit scoring does have its methodological limitations for measuring impacts of microcredit. We highlight five additional issues that researchers might consider when designing future studies.

First, theory suggests that spillovers, both positive and negative, may occur as a result of expanding access to credit. For example, expanding access to credit to some firms may harm their competitors and may help their suppliers or clients.

Measuring such spillovers requires a larger sample size than used here, and an enriched experimental design; for example, one might use two-stage randomization, first stratifying by geographic area or industry and then randomizing the proportion of each industry or geographic area that gets assigned to treatment. Second, many microlenders delegate credit decisions to borrowing groups. Credit scoring (with a random component) could, in principle at least, be provided to groups as an additional tool for use in deciding who gets credit and who does not. Third, new methods for mitigating underreporting of household and business debts may help researchers improve statistical power for estimating impacts of credit expansion on overall borrowing and more ultimate impacts. Fourth, having a loan application rejected may change future behavior (e.g., by discouraging pursuit of credit from another source). It is thus important to take care—as we did in the implementation studied here—to avoid changing the beliefs of rejected applicants (e.g., by rejecting people who are clearly creditworthy), lest the assignment to control (rejected) status induce behavior that differs from what people would have done in the absence of the experiment (39). Fifth, different methodologies may produce subject pools. For example, a branch expansion or clear loosening of credit criteria may draw in applicants previously discouraged from applying, in contrast to the methodology here, where the exact (weighting of) criteria that would affect a marginal application decision were opaque both before and after the credit-scoring intervention. Hence, our methodology identifies impacts on marginal borrowers only and may lack external validity for making inferences about inframarginal borrowers (who are clearly above or below the creditworthiness bar prior to the intervention) (40). This concern is mitigated in the present study because our cooperating lender deemed 74% of its first-time applicants “marginal.” From a policy perspective, marginal borrowers are often the subject pool of interest, even if the pool is smaller (proportionally) than it is here. If inframarginal borrowers are of interest as well, one practical methodological approach might be to introduce some randomness into the maximum loan size offered by the lender.

We conclude by recapping our two key points. One is that the theory and practice of microcredit remain far ahead of the evidentiary base needed to make good policy and to improve the delivery of financial intermediation. Our findings here are surprising and provocative: Microcredit in this context did not generate bigger businesses, higher income, and higher subjective well-being, but rather led to stronger risk management, fewer businesses, and lower subjective well-being. The current literature, and popular rhetoric from policymakers and microcredit institutions, puts forward a relatively simple story about microcredit working through business investment and female empowerment. In contrast, our findings suggest that microcredit works through more complex and disparate mechanisms that start with

the household rather than with the business. This leads us to our second point: The route to a clearer picture of whether and how microcredit works is methodological. Replicating sound research designs across different settings will help reveal whether treatment effects truly differ across settings, or whether the existing muddle is due more to methodological differences (and flaws). We hope we have convinced readers that the design used in this study is replicable, and we look forward to continued work with other lenders and researchers to build a better evidentiary base for microcredit policy and practice.

## References and Notes

- Note that whereas microcredit targets small businesses, the individuals may in practice use proceeds for household expenses, nonbusiness investments such as education or health, remittances, etc.
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- See (8) for additional details. Also note that the definition of “microcredit” is often debated, but typically includes loans to micro-entrepreneurs that are small but sufficiently large to provide meaningful support to small vendors, convenience stores or production facilities. Standard definitions often exclude “consumer” loans made to salaried individuals.
- Banerjee *et al.* (11) is the first randomized evaluation of group-liability micro-entrepreneurial microcredit, and Karlan and Zinman (12) reports on the first randomized evaluation of consumer lending. The key difference between Karlan and Zinman (12) and this paper is that the lender here targets entrepreneurs whereas the South African lender in the prior study was closer to a payday lender, making loans to employed individuals without any questions or examination of how funds would be used.
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- The USAID contractor is Chemonics’ Microenterprise Access to Banking Services (MABS). MABS provides technical assistance on bank operations, including advice on how to set prices in accordance with normal (i.e., not subsidized) consumer banking practices. MABS provides neither subsidized credit nor incentives to lower interest rates.
- Opportunity sets refer to investment opportunities, broadly defined. For example, a concern with nonrandomized studies is that those who get credit (because of relatively high demand or high supply) get it because they have businesses or other investment options with relatively high returns, and those who seem similar on observable characteristics but do not choose to borrow simply do not have the same set of business or investment options. A methodology that does not account for, or remove, this sort of underlying correlation may mistakenly attribute causal impacts to microcredit use/ access that are actually due to underlying differences between borrowers and nonborrowers that have nothing to do with microcredit per se. The same concern holds for other characteristics
- that are difficult to observe (and hence control for) that might be correlated with both outcomes (e.g., business success) and borrowing or lending decisions. The other examples in the text refer to what economists think of as “preferences” (e.g., patience or attitudes toward risk) and “aptitude” (e.g., entrepreneurial skills or business acumen).
- See (8) for a detailed discussion of the field experiments cited above and several other important microcredit evaluations using various methodologies (16–23).
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- The lender also requires first-time borrowers to open a savings account and maintain a minimum balance of 200 pesos. The real and nominal interest rates are roughly equivalent, as inflation in the Philippines during the study period was in the single digits.
- One branch was removed from the study when it was discovered that one account officer had found the underlying files saved by the credit-scoring software and altered both the assignment to treatment and data recorded from the application. This was discovered in audits of the proportion assigned to treatment, as well as audits to verify that the handwritten application from the client matched the data entered into the credit-scoring software. No other branches had problems revealed by such audits.
- “Business capacity” consists of business net cash flow, business assets, and number of employees. “Personal financial resources” consists of personal savings and credit history. “Outside financial resources” considers spousal employment and the value of household assets. “Stability” considers the ownership status and age of the business.
- Survey completion, is, unsurprisingly, correlated with baseline characteristics. Coupled with orthogonality to treatment assignment interacted with those same baseline characteristics, these results suggest that our results have internal validity but may lack external validity for those who did not complete surveys. See table S1 for details.
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38. However, as was the case in this study, operational considerations may dictate less than optimal experimental design regarding the proportion allocated to treatment versus control. Under normal circumstances and equal expected variance in treatment and control, power is optimized when treatment and control groups are equalized; however, the allocation rule in this study was not evenly split treatment and control groups because of portfolio management constraints.
39. Concerns about John Henry effects would affect estimation strategy: Treatment-on-the-treated estimates, using instrumental variables, would lead to biased estimates. Intent-to-treat estimates remain unbiased but make comparisons across settings more difficult if treatment compliance/take-up rates are quite heterogeneous.

40. Those with lower credit scores in our sample may be closer to the types of inframarginal applicants who might be encouraged to apply under a more visible expansion or loosening of credit. Tables S2 and S3 show little evidence of differential treatment effects for those with lower versus higher credit scores in our sample.

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1278/DC1  
Tables S1 to S4  
Data set and do files

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# Local Macrophage Proliferation, Rather than Recruitment from the Blood, Is a Signature of $T_H2$ Inflammation

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A defining feature of inflammation is the accumulation of innate immune cells in the tissue that are thought to be recruited from the blood. We reveal that a distinct process exists in which tissue macrophages undergo rapid *in situ* proliferation in order to increase population density. This inflammatory mechanism occurred during T helper 2 ( $T_H2$ )-related pathologies under the control of the archetypal  $T_H2$  cytokine interleukin-4 (IL-4) and was a fundamental component of  $T_H2$  inflammation because exogenous IL-4 was sufficient to drive accumulation of tissue macrophages through self-renewal. Thus, expansion of innate cells necessary for pathogen control or wound repair can occur without recruitment of potentially tissue-destructive inflammatory cells.

**R**ecruitment of leukocytes from the blood is the key feature of inflammatory responses to tissue damage or infection. Prominent in this cascade is the rapid influx of granulocytes and monocytes that subsequently differentiate into inflammatory macrophages and/or dendritic cells (1, 2). This paradigm of classical “type 1” inflammation is firmly grounded in processes elicited by microbial infection or necrotic cell death (2). Inflammation driven by helminth infection or allergy represents a distinct challenge to the immune system and is characterized by the recruitment of cells that produce the cytokine interleukin-4 (IL-4), including eosinophils, basophils, and  $CD4^+$  T helper 2 ( $T_H2$ ) cells (3, 4). Like classical inflammation, “type 2” inflammation also results in the accumulation of large numbers of macrophages in the affected tis-

sues (3, 5–7), yet despite this, IL-4 and  $T_H2$  responses are often regarded as “anti-inflammatory.”

A key distinguishing feature of macrophages in type 2 inflammation is their polarization toward an alternative (also known as M2) state of activation, mainly driven by IL-4 and IL-13 and characterized by expression of a distinct repertoire of molecules including arginase 1, resistin-like molecule alpha (RELM $\alpha$ ), and Ym1/2 (8). It has been widely assumed (1, 8) that accumulation of alternatively activated macrophages occurs through a process of recruitment similar to macrophage influx during classical inflammation. Supporting this view, depletion of blood monocytes impairs recruitment of mucosal M2 macrophages during gastrointestinal nematode infection (5). The intestinal environment presents unique challenges for the study of type 2-driven inflammation, however, because commensal bacteria may act as a classical trigger for monocyte influx.

**M2 macrophages accumulate independently of monocytes.** To examine type 2 inflammatory processes, we profiled cell recruitment during infection with a nematode that resides within “sterile” tissues (9). The rodent filarial nematode *Litomosoides sigmodontis* induces strongly  $T_H2$ -biased responses (fig. S1A) and drives alternative macrophage activation in the pleural cavity (Fig. 1A), after migrating there from the skin (Fig. 1B). For comparison, intrathoracic injection of thioglycollate was used as a benchmark of the clas-

sical inflammatory cascade (10), because when injected, it induces recruitment of macrophages to the pleural cavity. Indeed, thioglycollate injection resulted in the rapid influx of neutrophils ( $Gr-1^{high}/Ly-6C^{high}$ ) and  $Gr-1^{intermediate (int)}/Ly-6C^{high}$  monocytes, leading to accumulation of large numbers of  $F4/80^+$  macrophages by day 3 (Fig. 1C and fig. S1B). In contrast, *L. sigmodontis* infection triggered little neutrophil or  $Gr-1^{int}/Ly-6C^{high}$  monocyte recruitment to the pleural cavity during the first 15 days after infection (Fig. 1D and fig. S1C), even though larvae arrive at this site between 3 and 6 days after infection (Fig. 1B). Despite this, large numbers of  $F4/80^+$  macrophages gradually accumulated in the cavity after day 6 of infection (Fig. 1D), accompanied by their conversion to an alternatively activated phenotype (Fig. 1A). There was also a marked difference in surface phenotype of the macrophage populations elicited by nematode infection compared to classical inflammation: Those elicited by infection expressed levels of  $F4/80$  similar to that of resident  $F4/80^{high}$  macrophages from naïve tissues (fig. S1C), whereas those induced by thioglycollate expressed low  $F4/80$  levels (fig. S1B), which is characteristic of macrophages recruited to the serous cavities under inflammatory conditions (11).

To determine the role of monocytes in macrophage accumulation, we injected clodronate-loaded (CL) liposomes intravenously (iv). CL-liposomes block tissue infiltration by macrophages in a variety of inflammatory settings (5, 12, 13). This procedure depletes both  $Gr-1^{int}/Ly-6C^{high}$  monocytes, which are the precursors of  $F4/80^{low}$  inflammatory macrophages recruited during classical inflammation, and  $Gr-1/Ly-6C^-$  monocytes, which have been proposed as precursors of alternatively activated and tissue-resident macrophages (1, 14). As expected, treatment with CL-liposomes blocked the accumulation of macrophages in the pleural cavity induced by thioglycollate injection (Fig. 1E). In contrast, administration of CL-liposomes during the period when macrophages accumulate in the cavity (Fig. 1F), or when worms first enter the cavity (fig. S2), had no effect on either the number of pleural macrophages at day 10 after *L. sigmodontis* infection or the frequency of those expressing alternative activation markers. Macrophage accumulation occurred despite almost complete removal of  $Gr-1/Ly-6C^-$  monocytes from the blood

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throughout the entire period after CL-liposome treatment and transient depletion of Gr-1<sup>int</sup>/Ly-6C<sup>high</sup> monocytes (Fig. 1G).

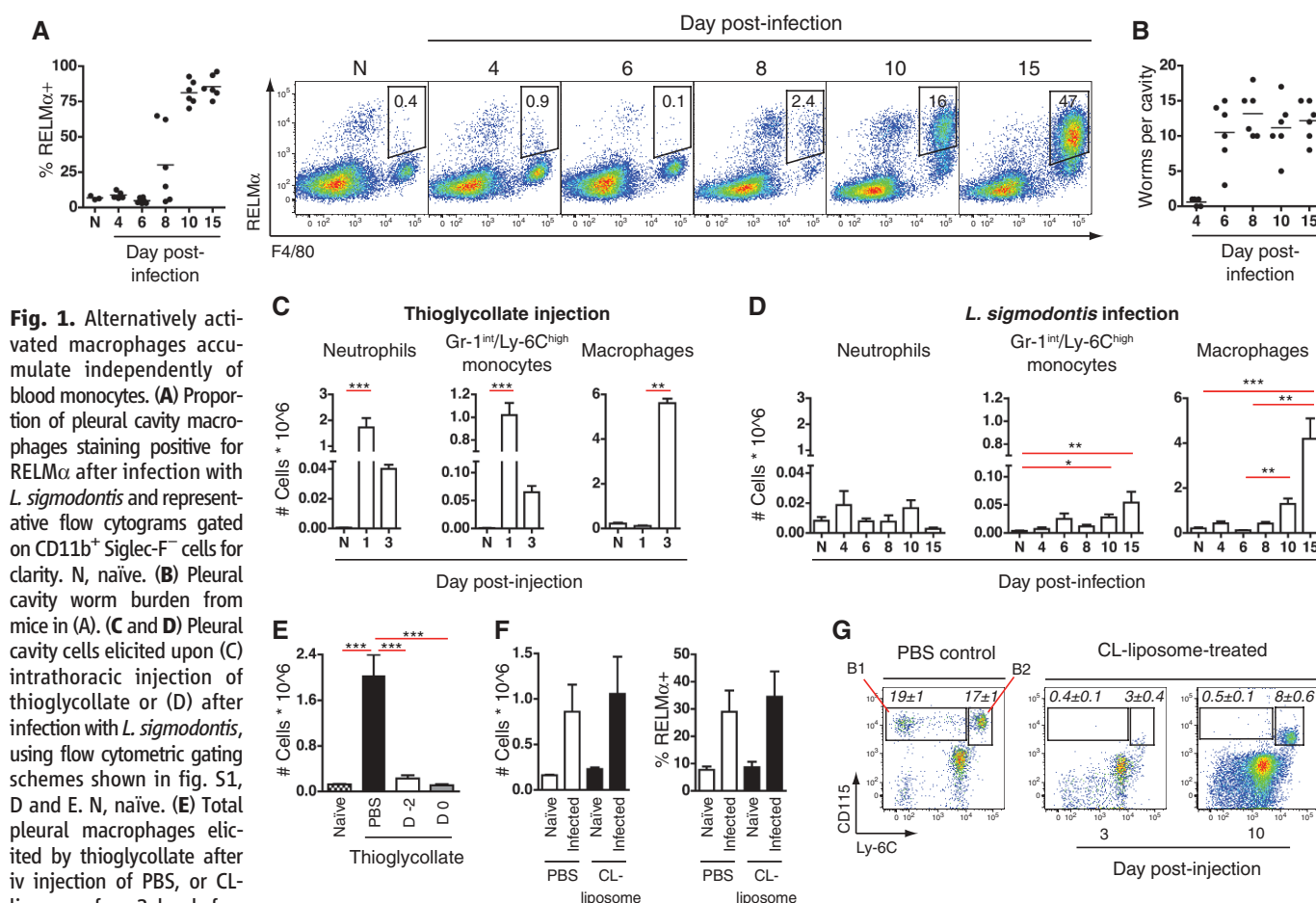
**Macrophages proliferate in situ.** These data raised the possibility that the large increase in macrophages seen during infection was the result of expansion of the resident population rather than blood cell recruitment. Although terminal differentiation of most mammalian cells typically results in reduced proliferative capacity, there is evidence that cells of the mononuclear phagocyte system can self-replicate in the periphery during development (15) or to maintain the tissue-resident population (16, 17), but not as a classical inflammatory mechanism. Accordingly, on average 17% of resident pleural macrophages in naïve mice were positive for Ki67, a marker of proliferation expressed throughout the cell cycle, and 1% were in S phase, as measured by a 3-hour pulse of 5-bromo-2'-deoxyuridine (BrdU) (Fig. 2, A and B, and fig. S3A). In contrast, less than 2% of thioglycollate-elicited macrophages expressed Ki67 and only 0.2% were in S phase (Fig. 2A). Notably, pleural macrophages isolated 10 days

after infection with *L. sigmodontis* exhibited a substantial increase in the frequency of dividing cells. On average, 38% of cells in the pleural cavity were positive for Ki67 and 6% were in S phase (Fig. 2B). BrdU<sup>+</sup> macrophages seen in the pleural cavity were not recruited from the blood because no blood monocytes incorporated BrdU in this 3-hour period (fig. S3B).

To determine whether macrophage proliferation occurs in a distinct type 2 setting, we looked in the peritoneal cavity after surgical implantation of the human parasitic filarial nematode *Brugia malayi*. Incision and suture of the abdominal wall without parasite implantation was used to control for the surgical procedure and provides a model of incisional wounding that is characterized by IL-4R $\alpha$ -dependent alternative macrophage activation (18). Both implantation of *B. malayi* and surgery alone resulted in an increased frequency of BrdU<sup>+</sup> and Ki67<sup>+</sup> peritoneal macrophages, although this was only maintained after parasite infection (fig. S4), when Th2 cells become activated (18). Ki67<sup>+</sup> macrophages have also been observed in the lungs of hookworm-infected mice

(7), and epidermal Langerhans cells up-regulate proliferation during atopic dermatitis (15), supporting the notion that macrophage proliferation is a characteristic common to various type 2 inflammatory settings.

**IL-4 instructs proliferative expansion.** IL-4 is important for macrophage accumulation during numerous helminth infections (5, 6). We thus infected IL-4-deficient mice with *L. sigmodontis* to test the requirement for IL-4 in proliferative expansion of tissue macrophages. Significantly fewer macrophages from IL-4-deficient mice were capable of proliferating, as determined by BrdU incorporation, leading to a failure of these cells to accumulate in the pleural cavity (Fig. 2C). As expected, macrophages that were present in IL-4-deficient mice were also unable to become alternatively activated, whereas worm burdens were indistinguishable between strains at this early stage of infection, as previously described (19). To demonstrate that IL-4 drives macrophage proliferation in vivo, we injected mice intraperitoneally (ip) with recombinant IL-4 complexed to antibody against IL-4 (IL-4c), which



**Fig. 1.** Alternately activated macrophages accumulate independently of blood monocytes. (A) Proportion of pleural cavity macrophages staining positive for RELM $\alpha$  after infection with *L. sigmodontis* and representative flow cytograms gated on CD11b<sup>+</sup> Siglec-F<sup>+</sup> cells for clarity. N, naïve. (B) Pleural cavity worm burden from mice in (A). (C and D) Pleural cavity cells elicited upon (C) intrathoracic injection of thioglycollate or (D) after infection with *L. sigmodontis*, using flow cytometric gating schemes shown in fig. S1, D and E. N, naïve. (E) Total pleural macrophages elicited by thioglycollate after iv injection of PBS, or CL-liposomes from 2 days before (D -2) or on the day of (D 0) thioglycollate injection. (F) Total pleural macrophages and the proportion producing RELM $\alpha$  on day 10 after *L. sigmodontis* infection after daily iv injection of CL-liposomes or PBS from days 6 to 9. (G) Frequency of Gr-1<sup>int</sup>/Ly-6C<sup>high</sup> CD115<sup>+</sup> (B1) or Gr-1<sup>int</sup>/Ly-6C<sup>high</sup> CD115<sup>+</sup> (B2) monocytes in CD11b<sup>+</sup> blood leukocytes on day 3 and 10 after *L. sigmodontis* infection after daily iv injection of PBS or CL-liposomes from days 2 to 5. Values in italics

represent the mean  $\pm$  SEM of eight mice. (A to G) All results are representative of two or three independent experiments. Graphs present mean  $\pm$  SEM of three to eight mice. (C and D)  $^*P < 0.05$ ,  $^{**}P < 0.01$ , and  $^{***}P < 0.001$ , as determined using Kruskal-Wallis test, with Dunn's post-test comparing all groups. (E)  $^{***}P < 0.001$ , as determined using one-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons.



increases the bioactive half-life of the cytokine (20). Delivery of IL-4c to naïve mice increased peritoneal cellularity over a 4-day period that was due primarily to increased macrophage numbers, all of which exhibited an alternative activation phenotype (Fig. 3A). IL-4-elicited macrophages expressed high levels of F4/80 [mean fluorescence intensity (MFI) of  $7300 \pm 680$  versus  $4900 \pm 380$  in phosphate-buffered saline (PBS)-treated controls; mean  $\pm$  SEM], and neither inflammatory monocytes nor neutrophils were recruited (fig. S5), consistent with a process distinct from classical inflammation (2, 11). Critically, the rate of macrophage proliferation was increased compared to mice receiving PBS alone (Fig. 3A), whereas no proliferation or accumulation was observed in IL-4 $\alpha$ -deficient mice (fig. S6). Thus, delivery of exogenous IL-4 was able to mimic the induction of proliferation and accumulation of macrophages observed during type 2 infection.

Macrophage proliferation in response to IL-4c was not restricted to the peritoneal cavity. There was an increase in number and frequency of Kupffer cells in the liver (Fig. 3B), as previously shown (20), and in the number of macrophages in the pleural cavity (Fig. 3C), and both of these populations exhibited increased incorporation of BrdU (Fig. 3, B and C). Thus, IL-4 can induce tissue macrophage proliferation in diverse body tissues, and we propose that in an environment with high IL-4 concentrations, the tissue macrophage population will undergo extensive proliferation.

### Self-renewal of resident tissue macrophages.

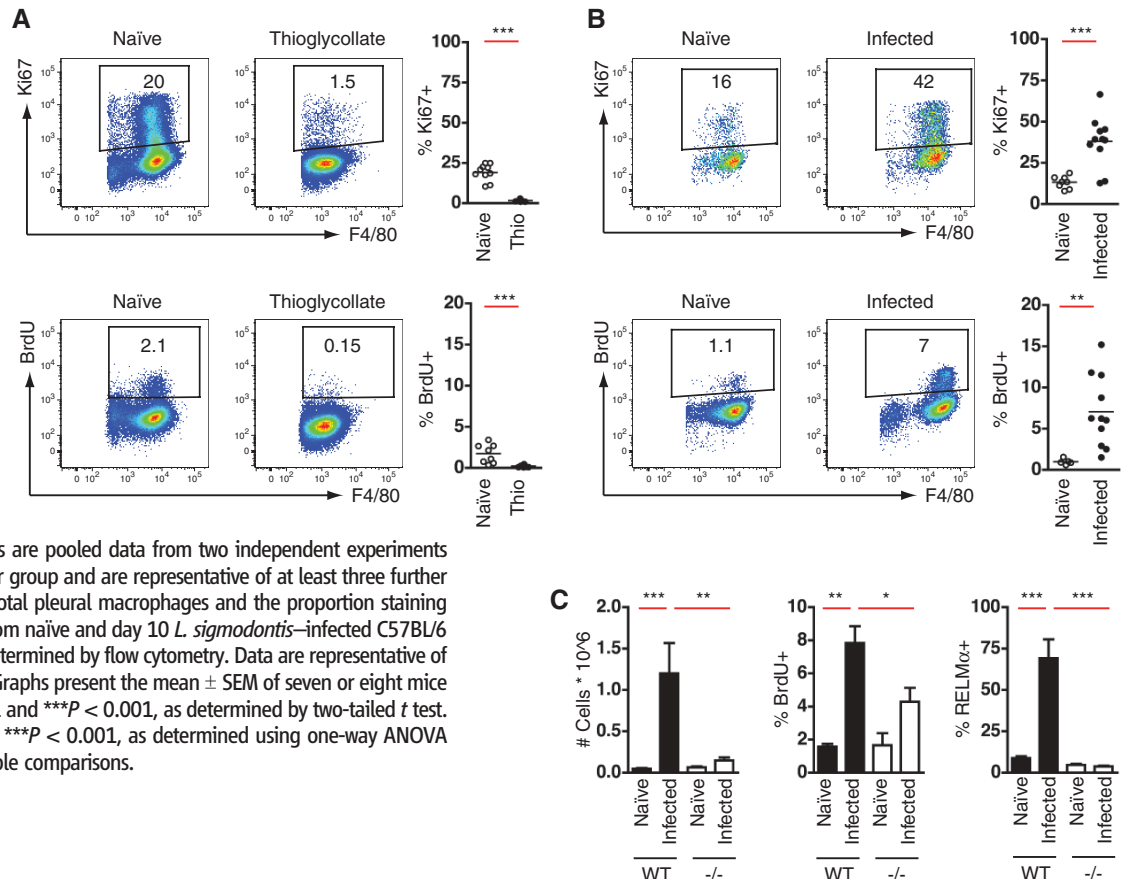
To establish definitively whether IL-4-driven accumulation of tissue macrophages is due to enhanced turnover of the resident population, we used a modified technique for creating bone marrow chimeric animals (21). Engraftment of bone marrow cells into lethally irradiated mice results in the replacement of most resident peritoneal macrophages by cells from transplanted bone marrow (17, 21). In the modified protocol, the upper body of *Cd45.1* congenic mice was shielded during irradiation to protect resident pleural macrophages from harmful effects of direct exposure while allowing engraftment of donor CD45.2<sup>+</sup> bone marrow. Mice were reconstituted for 8 weeks before ip injection of IL-4c or PBS, or intrathoracic injection of thioglycollate. As expected, partial shielding led to a mixed chimerism in peripheral blood monocytes (Fig. 3D) and granulocytes (fig. S7) in all groups of mice, with ~25 to 30% of donor origin. In contrast, only 3% of pleural macrophages were of donor origin in PBS-injected mice (Fig. 3D), which demonstrated that little replacement of these cells occurred from the bone-marrow under steady-state conditions. Despite a threefold increase in cell number, the same low-level chimerism (~3% donor-derived cells) was observed in pleural cavity macrophages of mice treated with IL-4c (Fig. 3D). Identical data were obtained for pleural macrophages from *L. sigmodontis*-infected chimeric mice (fig. S8). In contrast, macrophages elicited by injection of thioglycollate

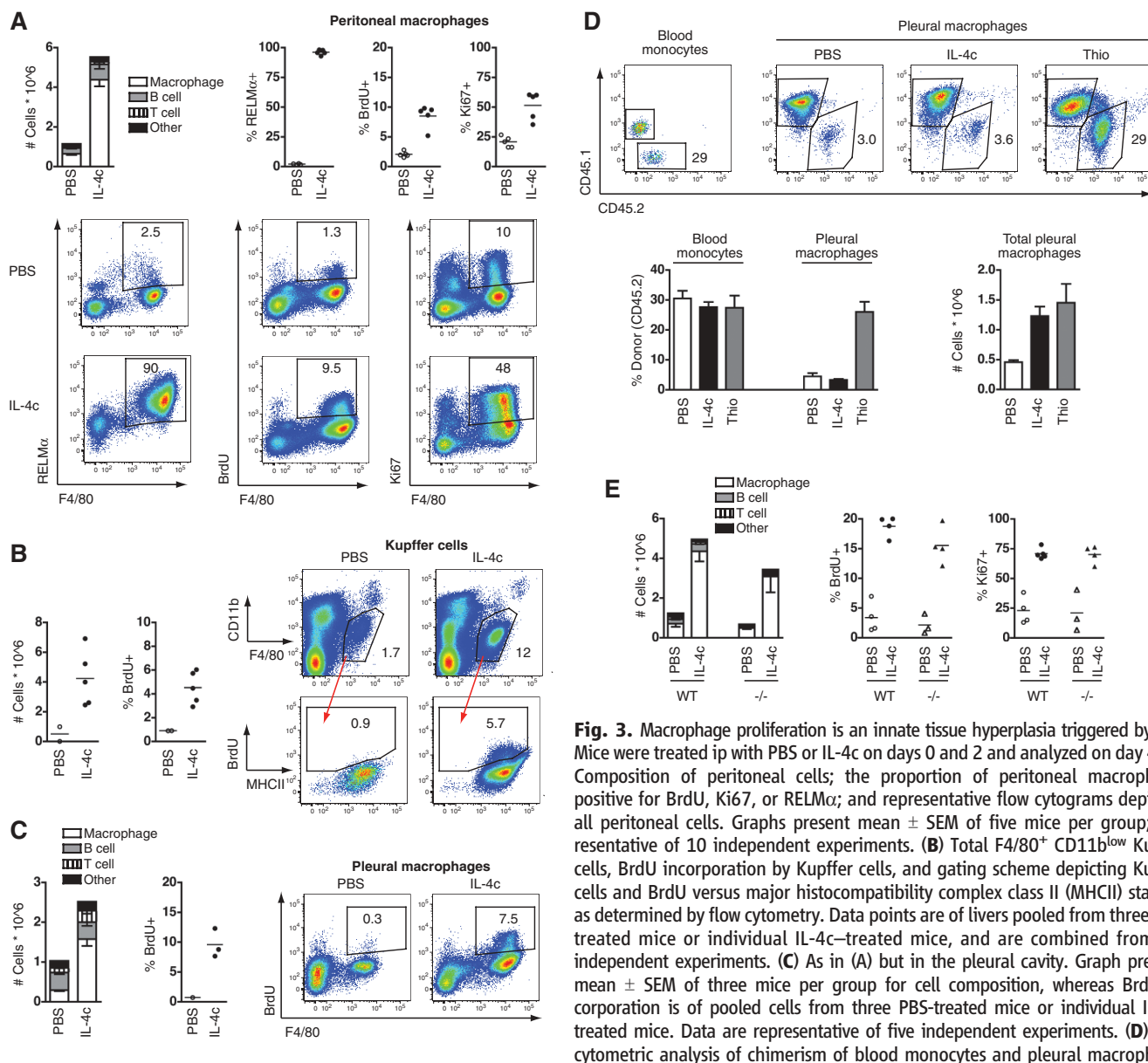
exhibited a high frequency of chimerism indistinguishable from that observed in blood monocytes and consistent with their differentiation from newly recruited cells of bone-marrow origin (Fig. 3D). These data reveal that IL-4 and filarial nematode infection elicit macrophages by expansion of the tissue-resident population, independent of recruitment of bone marrow-derived precursor cells.

Type 1 and type 2 immunity represent ancient innate pathways with fundamentally different purposes; one controls microbial pathogens (22) and the other macroparasites (23). Profound differences in amino acid utilization (24) and energy metabolism (25) of polarized macrophage populations illustrate the broad divergence of these pathways. Similarly, the IL-4-driven process of proliferative expansion described here contrasts sharply with the well-defined classical inflammatory process of recruitment (2) that predominates during type 1 infections (22). The capacity for IL-4-driven proliferation to achieve cell numbers similar to those of classical cell recruitment (Fig. 3C) suggests that this is a fundamental alternative inflammatory pathway. To verify that rapid proliferative expansion of macrophages represents an innate mechanism of inflammation, we injected RAG-1-deficient mice with IL-4c. Just as thioglycollate-induced macrophage recruitment occurs normally in RAG-1-deficient mice (18), IL-4c-mediated proliferative expansion was unaltered (Fig. 3E). These data reveal that accelerated

**Fig. 2.** Type 2 inflammation drives in situ proliferation of tissue macrophages.

(A) Flow cytometric analysis of Ki67 expression or BrdU incorporation by pleural macrophages from naïve mice or 3 days after thioglycollate (Thio) injection. BrdU was administered 3 hours before analysis. Gating schemes for Ki67 and BrdU analysis are shown in figs. S1B and S3A, respectively. Results are pooled data from two independent experiments with between 8 and 11 mice per group. (B) As in (A), but BrdU injection and analysis of cells were performed on day 10 after infection with *L. sigmodontis*. Results are pooled data from two independent experiments with between 5 and 11 mice per group and are representative of at least three further independent experiments. (C) Total pleural macrophages and the proportion staining positive for BrdU and RELM $\alpha$  from naïve and day 10 *L. sigmodontis*-infected C57BL/6 (WT) and *Il4*<sup>-/-</sup> (-/-) mice, as determined by flow cytometry. Data are representative of two independent experiments. Graphs present the mean  $\pm$  SEM of seven or eight mice per group. (A and B) \*\**P* < 0.01 and \*\*\**P* < 0.001, as determined by two-tailed *t* test. (C) \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001, as determined using one-way ANOVA with Tukey's post-test for multiple comparisons.

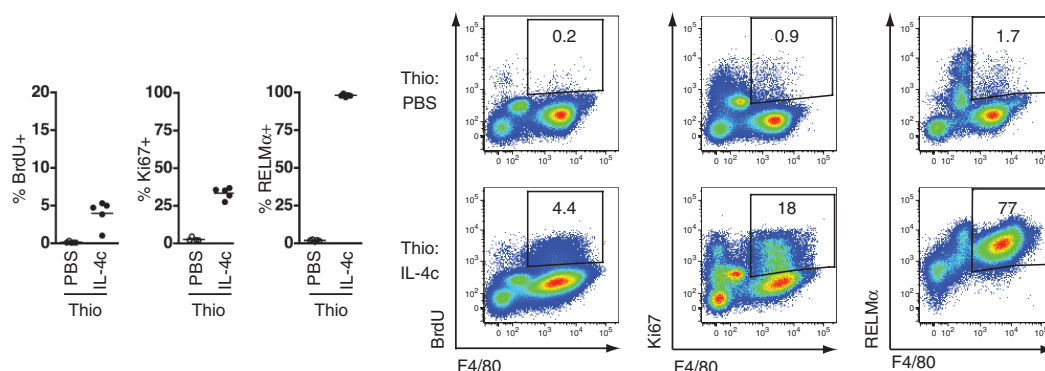




**Fig. 3.** Macrophage proliferation is an innate tissue hyperplasia triggered by IL-4. Mice were treated ip with PBS or IL-4c on days 0 and 2 and analyzed on day 4. **(A)** Composition of peritoneal cells; the proportion of peritoneal macrophages positive for BrdU, Ki67, or RELM $\alpha$ ; and representative flow cytograms depicting all peritoneal cells. Graphs present mean  $\pm$  SEM of five mice per group; representative of 10 independent experiments. **(B)** Total F4/80 $^{+}$  CD11b $^{low}$  Kupffer cells, BrdU incorporation by Kupffer cells, and gating scheme depicting Kupffer cells and BrdU versus major histocompatibility complex class II (MHCII) staining as determined by flow cytometry. Data points are of livers pooled from three PBS-treated mice or individual IL-4c-treated mice, and are combined from two independent experiments. **(C)** As in **(A)** but in the pleural cavity. Graph presents mean  $\pm$  SEM of three mice per group for cell composition, whereas BrdU incorporation is of pooled cells from three PBS-treated mice or individual IL-4c-treated mice. Data are representative of five independent experiments. **(D)** Flow cytometric analysis of chimerism of blood monocytes and pleural macrophages

and number of pleural macrophages in *Cd45.1* congenic mice given *CD45.2* $^{+}$  bone marrow cells after partial-body irradiation, and subsequently treated with PBS or IL-4c ip, or intrathoracic thioglycollate at week 8 after reconstitution. Representative *CD45.1* and *CD45.2* staining gated on *CD11b* $^{+}$  *CD115* $^{high}$  blood monocytes or *F4/80* $^{+}$  pleural macrophages. Graphs depict mean  $\pm$  SEM of four mice per group; data are representative of two independent experiments. **(E)** As in **(A)** but in C57BL/6 (WT) and *Rag1* $^{-/-}$  ( $-/-$ ) mice. Graphs depict mean  $\pm$  SEM of three or four mice per group; data are representative of two independent experiments.

**Fig. 4.** IL-4 triggers inflammatory macrophages to proliferate and alternatively activate. The proportion of peritoneal macrophages positive for BrdU, Ki67, or RELM $\alpha$  is shown, and representative flow cytograms depicting all peritoneal cells, from mice injected ip simultaneously with thioglycollate and PBS or IL-4c. IL-4c and PBS injections were repeated on day 2, and analysis was performed on day 4. Data are representative of two independent experiments, with five mice per group.





macrophage turnover is an innate response to IL-4, analogous to the classical inflammatory response to proinflammatory cytokines. In natural settings, the source of IL-4 will depend on the location and stage of type 2 inflammation and can be innate IL-4-producing cells or adaptive T<sub>H</sub>2 cells, as illustrated previously using the surgical wounding or *B. malayi* peritoneal implant models, respectively (18).

**Proliferation of recruited tissue macrophages.** Classical inflammatory stimuli such as microbial insult frequently colocalize with IL-4 production. To assess the effect of IL-4 in a setting where macrophages are actively recruited by classical stimuli, we simultaneously injected thioglycollate and IL-4c into the peritoneal cavity. Injection of thioglycollate in the presence of IL-4 induced the accumulation of macrophages expressing low levels of F4/80 (fig. S9), indicative of a recruited monocyte-derived population (11). In a separate experiment, proliferating M2 macrophages elicited by intrapleural injection of thioglycollate in combination with IL-4c ip were confirmed to be of blood origin through use of the modified bone marrow chimeric animals described earlier (fig. S10) (21). Critically, thioglycollate-elicited monocyte-derived macrophages could proliferate and become alternatively activated after treatment with IL-4 (Fig. 4 and fig. S10). Such a situation of mixed recruitment and expansion is likely to apply during infection with gastrointestinal nematodes where, despite evidence of recruitment from the blood, the total number of macrophages that accumulate in the gut mucosa is far reduced in IL-4-deficient mice (5). Collectively, our data demonstrate that both resident and recruited macrophages can alternatively activate and be driven to proliferate by a T<sub>H</sub>2 environment in vivo. Thus, there is neither a specific precursor for M2 macrophages, nor is proliferative capacity restricted by lineage.

**Two modes of inflammation.** The obvious benefit of classical inflammation is the rapidity with which large numbers of macrophages can accu-

mulate in the tissues, but the maintenance of a circulating precursor pool throughout the body is energetically costly. In contrast, the slower mechanism of local expansion in type 2 inflammation does not require enhanced bone marrow activity (Fig. 3D) and may deliberately avoid inflammatory cell recruitment and the associated potential for tissue damage. Indeed, IL-4 can actively block granulocyte recruitment during delayed-type hypersensitivity (26) and instruct macrophages to down-regulate production of proinflammatory chemotactic factors (27). This would be of particular benefit during wound healing, for example, where type 2-activated macrophages accelerate repair (23, 28, 29) and sustained classical inflammation results in a failure to heal (30, 31). Thus, we propose that proliferation in situ is an alternative mechanism of inflammation that allows macrophages to accumulate in sufficient numbers to perform critical functions such as parasite sequestration or wound repair in the absence of potentially damaging cell recruitment. This has broad therapeutic implications in settings where T<sub>H</sub>2-driven macrophages have key positive or negative function, such as in wound healing, autoimmunity, or the tumor microenvironment.

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# Real-Time Dynamics of Quantized Vortices in a Unitary Fermi Superfluid

Aurel Bulgac,<sup>1\*</sup> Yuan-Lung Luo,<sup>1</sup> Piotr Magierski,<sup>1,2</sup> Kenneth J. Roche,<sup>1,3</sup> Yongle Yu<sup>4</sup>

We introduce a comprehensive theoretical framework for the fermionic superfluid dynamics, grounded on a local extension of the time-dependent density functional theory. With this approach, we describe the generation and the real-time evolution and interaction of quantized vortices, the large-amplitude collective modes, as well as the loss of superfluidity at high flow velocities. We demonstrate the formation of vortex rings and provide a microscopic description of the crossing and reconnection of quantized vortex lines in a fermion superfluid, which provide the mechanism for the emergence of quantum turbulence at very low temperatures. We observe that superfluidity often survives when these systems are stirred with velocities far exceeding the speed of sound.

**S**uperfluidity and superconductivity are manifestations of quantum coherence at a macroscopic scale that have been observed in a

large range of physical systems: electron superconductivity, in liquid <sup>3</sup>He and <sup>4</sup>He and their mixtures, in nuclei and in neutron stars, and in

both fermionic and bosonic cold atoms in traps. Even though the critical temperatures at which a system undergoes a phase transition from a superfluid to a normal phase have a range approaching 20 orders of magnitude, properties of many of these systems are qualitatively similar and can be described within a common framework. One of the most successful phenomenological models is the two-fluid hydrodynamics (1, 2), where one assumes that the superfluid

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macrophage turnover is an innate response to IL-4, analogous to the classical inflammatory response to proinflammatory cytokines. In natural settings, the source of IL-4 will depend on the location and stage of type 2 inflammation and can be innate IL-4-producing cells or adaptive T<sub>H</sub>2 cells, as illustrated previously using the surgical wounding or *B. malayi* peritoneal implant models, respectively (18).

**Proliferation of recruited tissue macrophages.** Classical inflammatory stimuli such as microbial insult frequently colocalize with IL-4 production. To assess the effect of IL-4 in a setting where macrophages are actively recruited by classical stimuli, we simultaneously injected thioglycollate and IL-4c into the peritoneal cavity. Injection of thioglycollate in the presence of IL-4 induced the accumulation of macrophages expressing low levels of F4/80 (fig. S9), indicative of a recruited monocyte-derived population (11). In a separate experiment, proliferating M2 macrophages elicited by intrapleural injection of thioglycollate in combination with IL-4c ip were confirmed to be of blood origin through use of the modified bone marrow chimeric animals described earlier (fig. S10) (21). Critically, thioglycollate-elicited monocyte-derived macrophages could proliferate and become alternatively activated after treatment with IL-4 (Fig. 4 and fig. S10). Such a situation of mixed recruitment and expansion is likely to apply during infection with gastrointestinal nematodes where, despite evidence of recruitment from the blood, the total number of macrophages that accumulate in the gut mucosa is far reduced in IL-4-deficient mice (5). Collectively, our data demonstrate that both resident and recruited macrophages can alternatively activate and be driven to proliferate by a T<sub>H</sub>2 environment in vivo. Thus, there is neither a specific precursor for M2 macrophages, nor is proliferative capacity restricted by lineage.

**Two modes of inflammation.** The obvious benefit of classical inflammation is the rapidity with which large numbers of macrophages can accu-

mulate in the tissues, but the maintenance of a circulating precursor pool throughout the body is energetically costly. In contrast, the slower mechanism of local expansion in type 2 inflammation does not require enhanced bone marrow activity (Fig. 3D) and may deliberately avoid inflammatory cell recruitment and the associated potential for tissue damage. Indeed, IL-4 can actively block granulocyte recruitment during delayed-type hypersensitivity (26) and instruct macrophages to down-regulate production of proinflammatory chemotactic factors (27). This would be of particular benefit during wound healing, for example, where type 2-activated macrophages accelerate repair (23, 28, 29) and sustained classical inflammation results in a failure to heal (30, 31). Thus, we propose that proliferation in situ is an alternative mechanism of inflammation that allows macrophages to accumulate in sufficient numbers to perform critical functions such as parasite sequestration or wound repair in the absence of potentially damaging cell recruitment. This has broad therapeutic implications in settings where T<sub>H</sub>2-driven macrophages have key positive or negative function, such as in wound healing, autoimmunity, or the tumor microenvironment.

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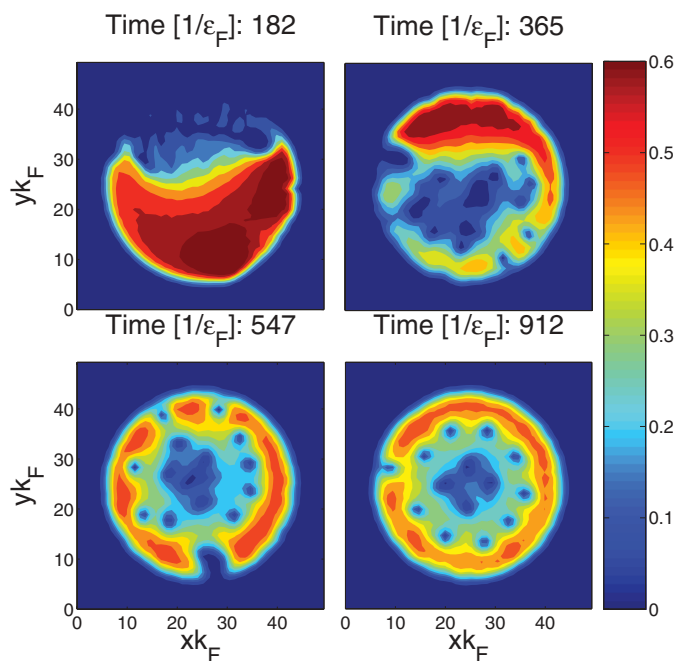


liquid  $^4\text{He}$  behaves like a mixture of normal and superfluid components. A notable property of superfluids is their ability to sustain quantized vortex lines (3, 4), which, unlike classical hydrodynamical vortices, have a quantized velocity circulation. With the use of the Ginzburg-Landau (GL) phenomenological theory (5), Abrikosov predicted that many quantized vortices self-organize into a triangular lattice (6). For a weakly interacting Bose gas in the superfluid phase, one can derive an approximate description, formally identical to the GL equation: the Gross-Pitaevskii (GP) equation (7, 8). In excited superfluids, vortices have a complicated time evolution; they cross and reconnect, a process extensively studied in Bose superfluids, which leads to quantum turbulence (9) and which, unlike turbulence in classical hydrodynamics, is realized basically in the absence of dissipation.

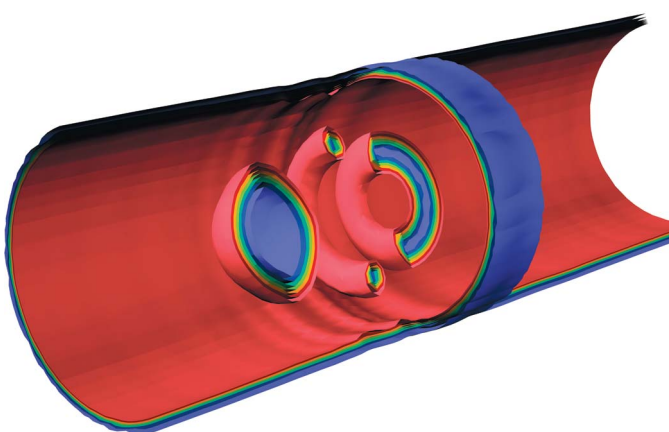
In spite of their success, traditional theoretical approaches fail to correctly describe a large class of phenomena in superfluids. Planck's constant does not appear in the two-fluid hydrodynamics formulation and thus, even though a quantized vortex is allowed to exist as a solution of these equations of motion, there is no intrinsic mechanism preventing quantized vortices from dynamically evolving into nonquantized vortices, and there is no intrinsic mechanism leading to the formation of a quantized vortex. Another drawback is the absence of Landau's critical velocity (10), the flow velocity at which a superfluid starts spontaneously converting into a normal fluid. In a fermionic superfluid, the critical velocity is the minimum of the sound velocity and of the flow velocity at which Cooper pairs start breaking up. The GL equation, which describes the spatio-temporal evolution of the order parameter, can be used and justified if the superfluid order parameter is small in amplitude and the temperature is near the critical temperature (11). However, if a fermionic superfluid is subjected to a flow with a velocity larger than the critical velocity, either a part of the system or the whole system can become normal, and the order parameter vanishes in the corresponding region of space. This kind of transition from a superfluid to a normal phase is not captured within a GL approach, as unlike the two-fluid hydrodynamics, there is no normal component. The excitation of magnitude of the pairing field (Higgs-Anderson mode) and other related modes (12, 13) is an example of where these phenomenological approaches fail. The existence of these modes can be proven in exactly solvable models (13). They are extremely slow, with frequencies much lower than the pairing gap, and they typically have a very large amplitude. In Fermi systems, superfluidity can be lost by either the Cooper pair breaking or the loss of the phase coherence of the condensate. Our results show that the Cooper pair breaking is a crucial element in the dynamics, in particular when superfluidity is lost and also in the case of the Higgs-Anderson modes (12, 13), when the (local) equilibrium quasiparticle distribution is violated.

We will focus here on the real-time dynamics of the unitary Fermi gas (UFG) to address these aspects and demonstrate the existence of new qualitative phenomena. The UFG became an object of intense theoretical and experimental study barely a decade ago (14–16), and over the years it proved to be a system with truly remarkable properties. When the Many-Body X Challenge was formulated in 1999 (17), there were neither compelling theoretical arguments nor any experimental evidence that would point to the mere mechanical stability or instability of a UFG. The first ab initio calculation of the UFG ground-state properties (18) and the first experimental realization of a quantum degenerate cloud of cold fermionic  $^6\text{Li}$  atoms in an atomic trap (19) were the first incontrovertible theoretical and experimental arguments in this direction. The subsequent observation of the vortex lattice provided the conclusive experimental proof that this system is indeed superfluidic (20). It became clear relatively recently that the UFG has the highest Landau critical velocity of any known superfluid (27).

We have used the Hohenberg-Kohn approach to density functional theory (DFT) (22) and its time-dependent extension (23) to develop a highly accurate theoretical framework capable of explicitly treating the pairing correlations and the time-dependent (TD) phenomena in a UFG at zero temperature [see (24) and supplemental online material (SOM)]. This framework is known as the time-dependent superfluid local density approximation (TDSLDA). The case of a UFG is special, as dimensional arguments alone place strong limits on the form of the functional. Galilean invariance (invariance with respect to the local reference frame velocity) can be invoked to determine its dependence on currents, which emerge when time-dependent phenomena are considered [see (24) and SOM]. The solution of the TDSLDA equations, which look formally like TD Bogoliubov-de Gennes (BdG) equations, require a rather sophisticated use of leadership-class supercomputers (25). The numerical complexity of these equations was a serious stumbling block until now and only a single limited two-dimensional (2D) solution of TDBdG



**Fig. 1.** Contour profiles of the pairing field  $|\Delta(x,y,t)|$  (where  $x$ ,  $y$ , and  $t$  are the space-time coordinates) of a unitary Fermi gas in a cylindrical container, stirred with a uniformly rotating rod. The colors codify the magnitude of the pairing field in units of the Fermi energy.  $n(x,y,t)$  and  $\Delta(x,y,t)$  are depleted in the core of the UFG vortices [see (29) and SOM]. The phase of  $\Delta(x,y,t)$  changes by  $2\pi$  around a vortex core in Fermi superfluids; thus, each fermion carries an additional  $\hbar/2$  angular momentum (30), unlike Bose superfluids, where each boson carries an additional  $\hbar$  angular momentum.  $\epsilon_F$  and  $k_F$  are the Fermi energy and momentum, respectively.



**Fig. 2.** A spherical projectile flying along the symmetry axis leaves in its wake two vortex rings.

equations has been attempted so far (26). The full 3D solution of the TDSLDA equations amounts to numerically solving tens to hundreds of thousands of coupled TD nonlinear 3D partial differential equations. We will demonstrate that within the time-dependent extension of DFT, when the quasiparticle wave functions  $[u_{n,\sigma}(\vec{r},t), v_{n,\sigma}(\vec{r},t)]$  ( $n$ , quantum number;  $\sigma_{(\uparrow\downarrow)}$ , spin up and spin down, respectively;  $\vec{r}$ , spatial coordinates;  $t$ , time) satisfy the TDSLDA equations, all the limitations of the traditional approaches discussed above are eliminated. In the absence of a spin-orbit coupling, these equations acquire the form

$$i\hbar \frac{\partial u_{n\uparrow}(\vec{r},t)}{\partial t} = h_{\uparrow}(\vec{r},t)u_{n\uparrow}(\vec{r},t) + \Delta(\vec{r},t)v_{n\downarrow}(\vec{r},t) \quad (1)$$

$$i\hbar \frac{\partial v_{n\downarrow}(\vec{r},t)}{\partial t} = \Delta^*(\vec{r},t)u_{n\uparrow}(\vec{r},t) - h_{\downarrow}^*(\vec{r},t)v_{n\downarrow}(\vec{r},t) \quad (2)$$

and similar equations for the components  $[u_{n\downarrow}(\vec{r},t), v_{n\uparrow}(\vec{r},t)]$  ( $i^2 = -1$ ;  $\hbar$ , Planck's constant  $h$  divided by  $2\pi$ ). The single-particle Hamiltonian  $h_{\uparrow,\downarrow}(\vec{r},t)$  and the pairing field  $\Delta(\vec{r},t)$  are the appropriate functional derivative of the UFG energy density functional, which depends on the entire set of quasiparticle wave functions  $u_{n,\sigma}(\vec{r},t), v_{n,\sigma}(\vec{r},t)$ . For a UFG, the leading order contribution to  $h_{\uparrow,\downarrow}(\vec{r},t) \propto n^{2/3}(\vec{r},t)$  and  $\Delta(\vec{r},t) \propto \kappa(\vec{r},t)$ , where  $n(\vec{r},t)$  and  $\kappa(\vec{r},t)$  are the normal and anomalous densities [see (24) and SOM]. The interactions with various applied external fields are described by including the appropriate potentials in  $h_{\uparrow,\downarrow}(\vec{r},t)$  [see (24) and SOM].

In our simulations, we placed a UFG in its ground state in a cylindrical trap with periodic boundary conditions along its axis, essentially flat inside and with soft lateral walls. Various soft objects were inserted into the superfluid; their impenetrability was adiabatically changed while moving them through the fluid (fig. S4). The stirring occurred only for a relatively short time interval. In one set of theoretical experiments, we introduced a rod and stirred the fluid by rotating the stirrers

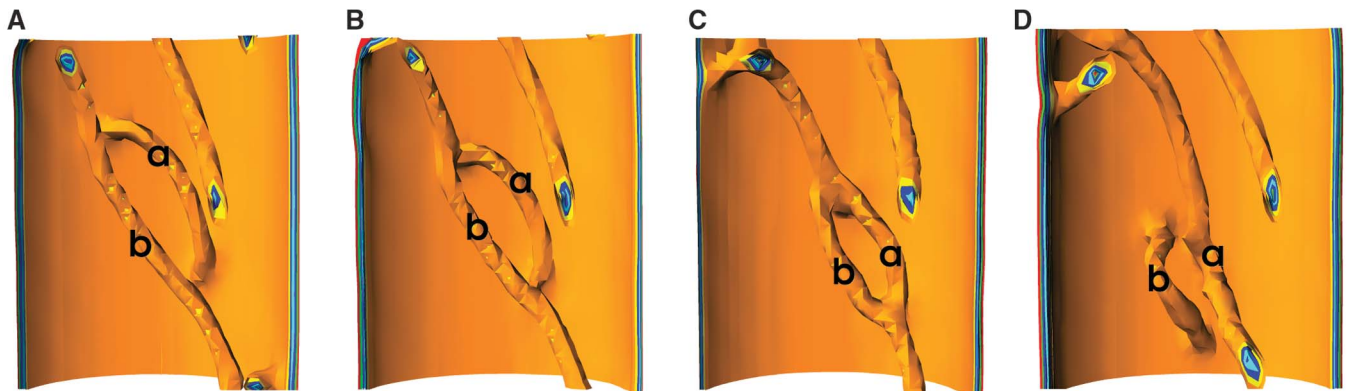
with constant angular frequency around the symmetry axis (Fig. 1 and SOM). In another set of simulations, we sent a spherical projectile along the axis of the trap and generated quantized vortex rings (Fig. 2 and SOM). We varied both the stirring radius  $R$  and the stirring angular frequency  $\omega$  and thus controlled the speed  $v_{\text{stir}} = R\omega$  of the stirrer(s). One can expect that if  $v_{\text{stir}} \ll v_c$ , where  $v_c$  is the UFG critical velocity, the system will probably return to its initial state after the stirring is turned off. However, if  $v_{\text{stir}} > v_c$ , one expects that the whole system or a part of it will become normal and the superfluid order parameter will vanish in the corresponding regions of space. For a stirring with speeds  $v_1 < v_{\text{stir}} < v_c$ , where  $v_1$  is some minimal geometry-dependent stirring velocity, it is natural to expect that one or more vortices will be generated. It is by studying such processes, as well as the Higgs-Anderson modes (12, 13), that one can ascertain the qualitative differences between the predictions of traditional approaches and TDSLDA. An obvious limitation of TDSLDA is the neglect of the so-called potential-energy surface hopping, and an extension of this approach is warranted to accurately describe the long-time dynamics (27).

Although it was expected to generate a relatively small number of vortices when the stirring velocity is low (and that the number of vortices increases with the stirring velocity), a number of features of the dynamic vortex generation are unexpected. The fact that the UFG is highly compressible results in very large time-dependent variations of the number density. Often the entire mass of the system is gathered in front of the stirrer, leaving little matter behind it, even at subsonic speeds. The gas occupies less than half of the available volume, even when the excluded volume by the stirrer is quite small, and it comes as a surprise that the system does not lose quantum coherence under such a violent perturbation. Moreover, the system organizes itself in an almost perfect vortex lattice after the stirring is turned off. Even more surprising is that the system remains a superfluid, even when stirred at supercritical speeds. We have observed that the system forms a vortex lattice even if stirred with

speeds up to  $v_{\text{stir}} = 0.65v_F > v_c \approx 0.365v_F$  (where  $v_F$  is the Fermi velocity) (see SOM). In a gas, unlike a liquid with a rather well-defined density, the increase of the density of the cloud during the stirring process leads to an increased local critical velocity and, thus, to a stable superflow at speeds larger than the nominal Landau's critical velocity.

Figure 2 illustrates the formation of vortex rings by a spherical projectile flying with velocity  $0.2v_F < v_c$  along the axis of a cylindrical trap. Two vortex rings are formed with slightly different radii; thus, they propagate with different drift velocities. After their interaction, these rings emerge with different radii. Similar processes have been studied for quite some time in the case of Bose superfluids using the GP equation (28). In Fig. 3, we demonstrate the microscopic dynamics of the reconnection of two vortex lines, the underlining mechanism of the quantum turbulence (9) in superfluids, where dissipation plays an unimportant role. In Fig. 3, two vortex lines cross at two points and exchange finite segments, a process reminiscent of DNA recombination. In all of the cases we studied, regular sound waves (phonons) were also excited. In a separate simulation of a sphere passing through the system along a line parallel to the axis (see SOM), we have observed that the recombination of two vortex lines is often a multistep process: Two vortex lines cross and then recross and rejoin several times before finally separating. In addition, when we generated vortices using a rod and a sphere (see SOM), the translational symmetry was destroyed and one could clearly observe the excitation of waves running along the vortex lines (Kelvin waves). With time, the Kelvin modes apparently transfer their energy to phonons from short length scales to larger length scales—a mechanism that is still controversial in the quantum turbulence community (9). This particular aspect requires further and more detailed analysis, as our imposition of periodic boundary conditions along the third axis might have played a role in facilitating this behavior.

The various excitation scenarios of a UFG illustrated here demonstrate the power of this new framework, the richness of the phenomena



**Fig. 3.** (A to D) Two vortex lines approach each other, connect at two points, form a ring and exchange between them a portion of the vortex line, and subsequently separate. Segment (a), which initially belonged to the vortex line attached to the wall, is transferred to the long vortex line (b) after reconnection and vice versa.



that are still awaiting to be fully explored, and the extreme flexibility of TDSLDA. Because of the complexity of the full 3D TDSLDA equations, these phenomena have never been addressed, even at the mean-field level in fermionic superfluids. Within the TDSLDA framework, one can address with high accuracy on a microscopic basis the real-time dynamics of a UFG superfluid and study quantum turbulence at zero temperature, where dissipative processes are inhibited.

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## Supporting Online Material

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SOM Text

Figs. S1 to S7

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References

Movies S1 to S40 at [www.phys.washington.edu/groups/qmbnt/UFG/](http://www.phys.washington.edu/groups/qmbnt/UFG/)

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# Transformation Optics Using Graphene

Ashkan Vakil and Nader Engheta\*

Metamaterials and transformation optics play substantial roles in various branches of optical science and engineering by providing schemes to tailor electromagnetic fields into desired spatial patterns. We report a theoretical study showing that by designing and manipulating spatially inhomogeneous, nonuniform conductivity patterns across a flake of graphene, one can have this material as a one-atom-thick platform for infrared metamaterials and transformation optical devices. Varying the graphene chemical potential by using static electric field yields a way to tune the graphene conductivity in the terahertz and infrared frequencies. Such degree of freedom provides the prospect of having different "patches" with different conductivities on a single flake of graphene. Numerous photonic functions and metamaterial concepts can be expected to follow from such a platform.

The fields of plasmonics, metal optics, metamaterials, and transformation optics (1–8) offer a collection of techniques to tame the electromagnetic fields into desired spatial patterns, providing applications in engineering and applied sciences. Owing to their ability to support the surface-plasmon polariton (SPP) surface waves in the infrared and visible regimes, the noble metals, such as silver and gold, have been popular materials for constructing optical metamaterials (1). However, the difficulty in controlling and varying permittivity functions of noble metals and the existence of material losses—especially at visible wavelengths—degrade the quality of the plasmon resonance and limit the relative propagation lengths of SPP waves along the interface between such metals and dielectric materials.

These drawbacks constrain the functionality of some of metamaterials and transformation optical devices. We show that graphene, which has exciting electronic transport properties (9, 10) suitable for optoelectronic applications (11–16), may also serve as a platform for metamaterials and transformation optical devices.

Graphene's complex conductivity ( $\sigma_g = \sigma_{g,r} + i\sigma_{g,i}$ ) depends on the radian frequency  $\omega$ , charged particle scattering rate  $\Gamma$  representing the loss mechanism, temperature  $T$ , and chemical potential  $\mu_c$ . The chemical potential depends on the carrier density and can be controlled by gate voltage, electric field, magnetic field, and/or chemical doping (17, 18). The imaginary part of graphene conductivity can attain negative and positive values in different ranges of frequencies depending on the level of chemical potential (17). The complex conductivity of a free-standing isolated graphene at  $T = 3$  K, with  $\Gamma = 0.43$  meV (17)—computed from the Kubo formula, which is in agreement with experi-

mental results (18)—shows regions of frequencies and chemical potentials for which  $\sigma_{g,i} < 0$ , whereas for other regions  $\sigma_{g,i} > 0$  (Fig. 1, A and B). When  $\sigma_{g,i} > 0$ , a graphene layer effectively behaves as a very thin "metal" layer (19) capable of supporting a transverse-magnetic (TM) electromagnetic SPP surface wave (20–23). The dispersion relation of this TM SPP surface wave is expressed as  $\beta^2 = k_0^2[1 - (2/\eta_0\sigma_g)^2]$ , where  $\beta$  and  $k_0$  are, respectively, the wave numbers of the guided mode and the free space, and  $\eta_0$  is the intrinsic impedance of free space (19–22). However, when  $\sigma_{g,i} < 0$ , TM SPP guided surface wave is no longer supported by the graphene. Instead, a weakly guided transverse-electric (TE) electromagnetic SPP surface wave might be present (19–21). There are at least two major advantages for a graphene layer as compared with the noble metal surfaces: (i) As far as the SPP characteristics are concerned, in the mid infrared (IR) wavelengths the combination of two features of SPP—the propagation length, defined as  $1/\text{Im}(\beta)$ , and the mode lateral extent, proportional to approximately  $1/\text{Re}(\beta)$ —for graphene can be more favorable than for silver or gold (19); the real part of wave number  $\beta$  for the TM SPP wave along the graphene is much larger than the wave number of free space,  $k_0 \equiv \omega(\mu_0\epsilon_0)^{1/2}$  (fig. S1A) (19). As a result, such a SPP surface wave is tightly confined to the graphene layer, with guided wavelength ( $\lambda_{\text{SPP}}$ ) much smaller than free space wavelength  $\lambda_0$  ( $\lambda_{\text{SPP}} \ll \lambda_0$ ), whereas its imaginary part of wavenumber is small (22, 24). (ii) The most important advantage of graphene over thin metal layers and metal interfaces is the capability to dynamical-

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of chemical doping or gate voltage— $E_{\text{bias}}$  in real time, locally and inhomogeneously. By using different values of  $E_{\text{bias}}$  at different locations across the single graphene layer, we can create certain desired conductivity patterns.

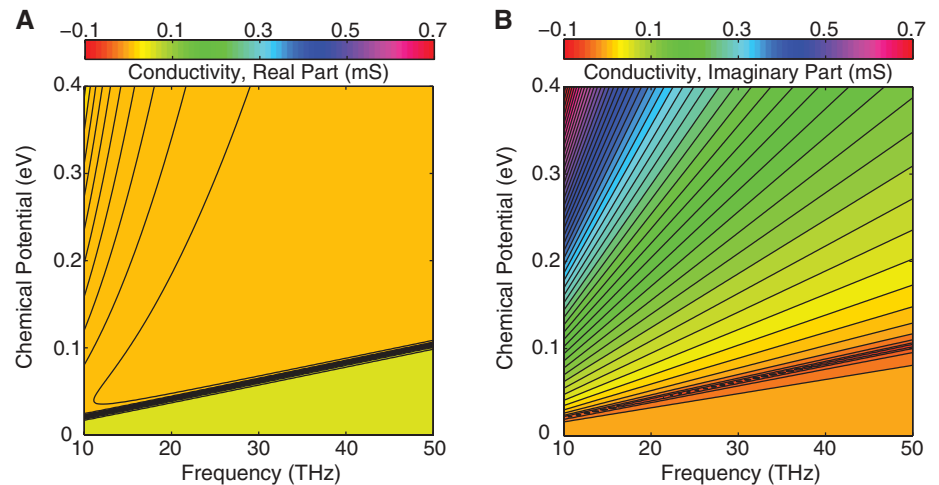
We present several scenarios in which the proper choice of conductivity spatial patterns across the graphene provides exciting possibilities for tailoring, manipulating, and scattering IR SPP wave signals across the graphene. To start, consider a SPP mode at 30 THz guided by a uniformly biased graphene layer (fig. S2) (19). The TM SPP-guided wavelength  $\lambda_{\text{SPP}}$  for this free-standing graphene in air at  $T = 3$  K with  $\Gamma = 0.43$  meV and  $\mu_c = 0.15$  eV is  $\lambda_0/69.34 = 144.22$  nm, with  $\text{Re}(\beta) = 69.34k_0$  and  $\text{Im}(\beta) = 0.71k_0$ . This highly compressed mode offers an effective SPP index ( $n_{\text{SPP}} \equiv \beta_{\text{SPP}}/k_0$ ) of 69.34 and has a relatively large propagation length of  $15.6\lambda_{\text{SPP}} = 0.225\lambda_0 = 2.25$   $\mu\text{m}$ . We can engineer the SPP surface wave to reflect and refract on this sheet of graphene (Fig. 2) by varying the electric field bias spatially. To achieve spatially varying static electric field bias, one may consider three possible methods: (i) a split gate structure to apply different bias voltages to different gates (Fig. 2). The potentials  $V_{b1}$  and  $V_{b2}$ , applied to two gate electrodes, are chosen to provide different chemical potential values, such as  $\mu_{c1} = 0.15$  eV and  $\mu_{c2} = 0.065$  eV, in the two halves of graphene. For the sake of clarity, in Fig. 2 the “gate electrodes” are symbolically shown above the graphene at a small distance. However, the gates may be located on the substrate beneath the graphene. (ii) An uneven ground plane (Fig. 3, A and B, and figs. S7 and S8). By designing the specific profile of the ground plane underneath the dielectric spacer holding the graphene, one can achieve nonuniform static biasing electric field under the graphene, resulting in different conductivity values at different segments of graphene (19). (iii) Inhomogeneous permittivity distribution near the top surface of the spacer holding a sheet of graphene (fig. S9) (19). Inhomogeneous distribution of permittivity generates a nonuniform static electric field under the graphene, creating inhomogeneous chemical potential and in turn inhomogeneous conductivity across the graphene (19).

In Fig. 2, the conductivity values of the two segments calculated from the Kubo formula with  $T = 3$  K and  $\Gamma = 0.43$  meV are, respectively,  $\sigma_{g1} = 0.0009 + i0.0765$  mS and  $\sigma_{g2} = 0.0039 - i0.0324$  mS. The “farther” half section with  $\sigma_{g1,i} > 0$  supports a TM SPP, whereas the “closer” half with  $\sigma_{g1,i} < 0$  does not. If a TM SPP is launched in the farther-half section toward the junction of two sections, it reflects back at that boundary line. Then, the incident and reflected SPP waves add up to form an oblique standing wave. The reflection of SPP at this line resembles the Fresnel reflection of a plane wave from a planar interface between two media. Here, however, such reflection occurs across a one-atom-thick platform, with a favorably little radiation loss owing to high confinement of SPP to the graphene. This case

might also be analogous to the Fresnel reflection from a planar interface between a medium that supports propagating waves (for example, a medium with a real refractive index, such as a dielectric) and another medium that does not support propagating waves (for example, a medium with no real index, such as a noble metal). Accordingly, on the graphene the Fresnel reflection of SPP results in a near complete reflection. The simulation results reveal an effective reflection at the boundary line between the two segments. In Fig. 2, a guided IR edge wave along the boundary line between the two sections can be observed (19). This phenomenon might be related to the electronic behavior near the p-n junction formed on the graphene (13, 14, 25).

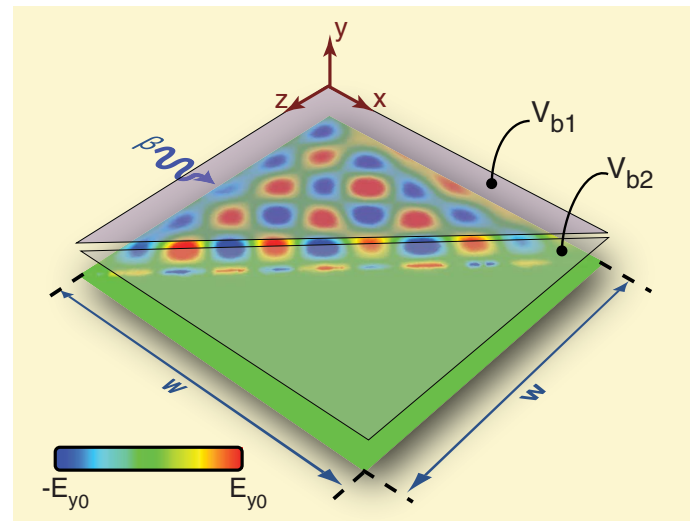
On the basis of the reflection mechanism mentioned above, we propose a setting analogous to a conventional three-dimensional (3D) metal-insulator-metal (MIM) waveguide, but here on a one-atom-thick platform (Fig. 3A). This 2D

waveguide variant consists of three distinct regions on the graphene: two side regions with chemical potential  $\mu_{c2}$  resulting in conductivity  $\sigma_{g2} = 0.0039 - i0.0324$  mS where  $\sigma_{g2,i} < 0$ , and a middle “ribbon-like” section, with chemical potential  $\mu_{c1}$  yielding conductivity  $\sigma_{g1} = 0.0009 + i0.0765$  mS where  $\sigma_{g1,i} > 0$ . An uneven ground plane, for example, can be used to achieve these two different chemical potentials (19). However, because the SPP is highly confined to the graphene, in all our numerical simulations the graphene is assumed to be free standing in vacuum (26). A guided SPP wave is bounded by the two boundary lines between the graphene sections with different conductivities, as shown in Fig. 3A. Moreover, an IR splitter, based on the waveguide concept, might be envisioned. This can be achieved by proper design of conductivity patterns on the graphene, generated, for example, by use of uneven ground plane or other techniques (19) to create required spatial distribution



**Fig. 1.** (A) Real part and (B) imaginary part of the conductivity as a function of the chemical potential and frequency ( $T = 3$  K,  $\Gamma = 0.43$  meV), following the Kubo formula (17).

**Fig. 2.** Simulation results showing snap shot in time of  $y$  component of the electric field,  $E_y$  for the near total reflection of a TM electromagnetic SPP wave on a free-standing graphene ( $w = 800$  nm,  $T = 3$  K,  $\Gamma = 0.43$  meV,  $\mu_c = 0.15$  eV). The  $V_{b1}$  and  $V_{b2}$  are chosen so that the two halves of graphene acquire complex conductivity values  $\sigma_{g1} = 0.0009 + i0.0765$  mS and  $\sigma_{g2} = 0.0039 - i0.0324$  mS.





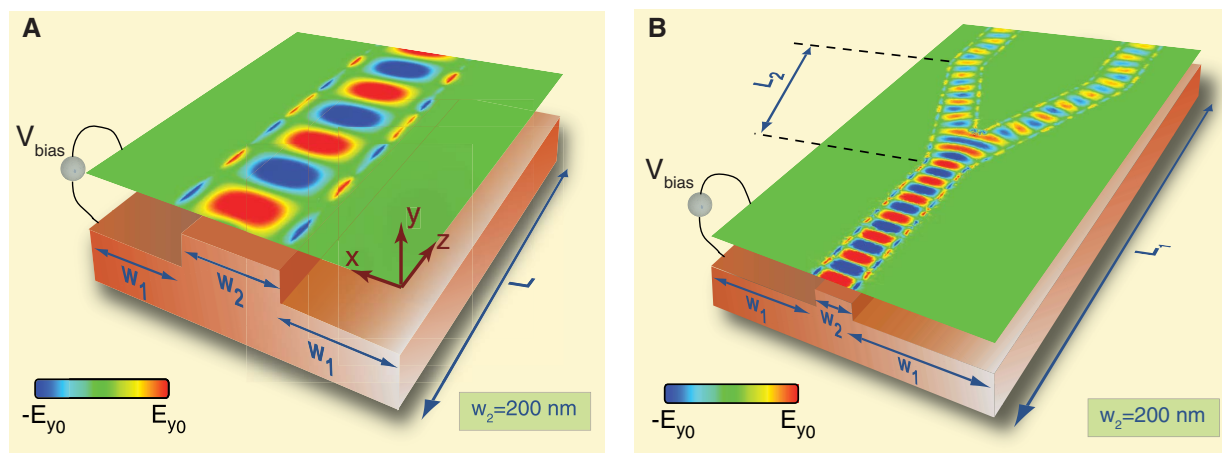
of the bias electric field (Fig. 3B). The mechanism for guiding IR signals across the graphene offers the possibility of contriving miniaturized, one-atom-thick photonic circuitry for information processing at the nanoscale (19, 27).

On the basis of the proposed concepts, one can also envisage 2D IR metamaterials and transformation optical devices on a single layer of carbon atoms. Figure 4A shows numerical simulation of SPP propagation along a free-standing layer of graphene, within which an array of 2D circular “patches” is created. These patches are biased at voltage  $V_{b2}$  ( $\sigma_{g2} = 0.0039 - i0.0324$  mS, where  $\sigma_{g2,i} < 0$ ), whereas the rest of graphene is biased at  $V_{b1}$  ( $\sigma_{g1} = 0.0009 + i0.0765$  mS, where  $\sigma_{g1,i} > 0$ ). Each circular patch acts as a scatterer for the SPP surface wave, behaving as a single-atom-layered “flatland inclusion.” The collection of these “inclusions” can result in a 2D bulk flat metamaterial. Our numerical simulations suggest that such geometry, if designed properly, can act as a 2D version of metamaterials formed by collection of

subwavelength metallic nanoparticles that may, under certain conditions, exhibit backward wave propagation effect (28). Furthermore, a “flat” version of a Luneburg lens, which is an example of transformation optical devices (29, 30), is presented in Fig. 4B. Such graphene-based Luneburg lens can be designed by creating several concentric rings with specific conductivity values. With a special configuration of bias arrangement, we can create, approximately, a graded conductivity pattern that provides the required effective SPP-graded index for operation of the Luneburg lens. To find the corresponding conductivities for these concentric rings (for a Luneburg lens with diameter  $D$ ), we used the discretized approximate expression  $\sigma_{i,n} = \sigma_{i,out} \{2 - [(r_n + r_{n-1})/D]^2\}^{-1/2}$  (19), where  $\sigma_{i,n}$  and  $r_n$  denote, respectively, the imaginary part of conductivity and radius of the  $n^{\text{th}}$  section, and  $\sigma_{i,out}$  is the imaginary part of conductivity of the background graphene. Our simulation results (Fig. 4B) reveal that the SPP generated from a “point-like” source

is evolved into an approximately one-atom-thick “collimated beam” of SPP on the graphene, as a conventional 3D Luneburg lens collimates wavefronts generated from a point source into a 3D beam. The diameter of the “flat” Luneburg lens is about  $1.5 \mu\text{m}$ , which is  $0.15\lambda_0$ —a notably subwavelength size. This example suggests that various subwavelength IR devices (such as convex lenses and concave lenses) can be designed on the graphene, providing a versatile platform for nanoscale Fourier optics and other photonic signal-processing methods.

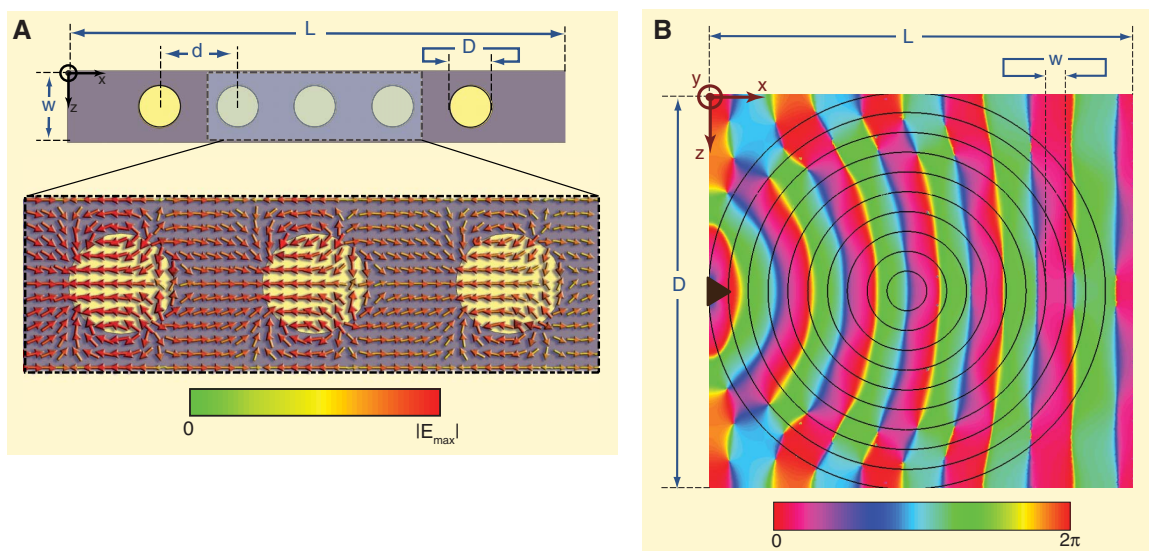
Our theoretical study of IR wave interaction with graphene suggests that the graphene can be a low-loss one-atom-thick platform for flatland IR metamaterials and transformative optics. The inhomogeneous, nonuniform patterning of conductivity may be achieved by various techniques, for example, by creating uneven ground plane, fabricating inhomogeneous permittivity of spacer dielectric, and applying gate electric or magnetic field.



**Fig. 3.** (A) Simulation results of  $E_y$  (snap shot in time) for an IR-guided wave at  $f = 30$  THz along the ribbon-like section of graphene with the chemical potential  $\mu_{c1}$  ( $L = 560$  nm,  $w = w_1 + w_2 + w_3 = 200 + 200 + 200$  nm). (B) Similar

to (A), but here the ribbon-like section splits into two paths ( $L_1 = 1077$  nm,  $L_2 = 560$  nm,  $w = w_1 + w_2 + w_3 = 600 + 200 + 600$  nm) (26). Different scale bars in each panel. The graphene conductivity parameters are similar to those in Fig. 2.

**Fig. 4.** (A) Flatland metamaterial: the snap shot in time of the electric field vectors for the TM SPP along a single sheet of graphene at  $f = 30$  THz, shown on the graphene plane. Only one row of the 2D periodic array is shown ( $D = 30$  nm,  $d = w = 55$  nm,  $L = 370$  nm). (B) One-atom-thick Luneburg lens: Simulation results showing the phase of  $E_y$  of the SPP at  $f = 30$  THz along the graphene ( $D = 1.5 \mu\text{m}$ ,  $w = 75$  nm,  $L = 1.6 \mu\text{m}$ ).



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- For example, for a layer of graphene free standing in air at  $T = 3$  K with  $\Gamma = 0.43$  meV and  $\mu_c = 0.15$  eV at frequency 30 THz,  $\text{Re}(\beta) = 69.34k_0$  and  $\text{Im}(\beta) = 0.71k_0$ . The corresponding numbers for the SPP at the air-silver interface in the mid IR wavelengths are approximately  $\text{Re}(\beta) \approx k_0$  and  $\text{Im}(\beta) < 10^{-4}k_0$ , resulting in a very weakly confined SPP.
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## Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S9

References

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# Wafer-Scale Graphene Integrated Circuit

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A wafer-scale graphene circuit was demonstrated in which all circuit components, including graphene field-effect transistor and inductors, were monolithically integrated on a single silicon carbide wafer. The integrated circuit operates as a broadband radio-frequency mixer at frequencies up to 10 gigahertz. These graphene circuits exhibit outstanding thermal stability with little reduction in performance (less than 1 decibel) between 300 and 400 kelvin. These results open up possibilities of achieving practical graphene technology with more complex functionality and performance.

Graphene, a layer of carbon atoms arranged in a hexagonal lattice, is a promising candidate for future high-speed electronics

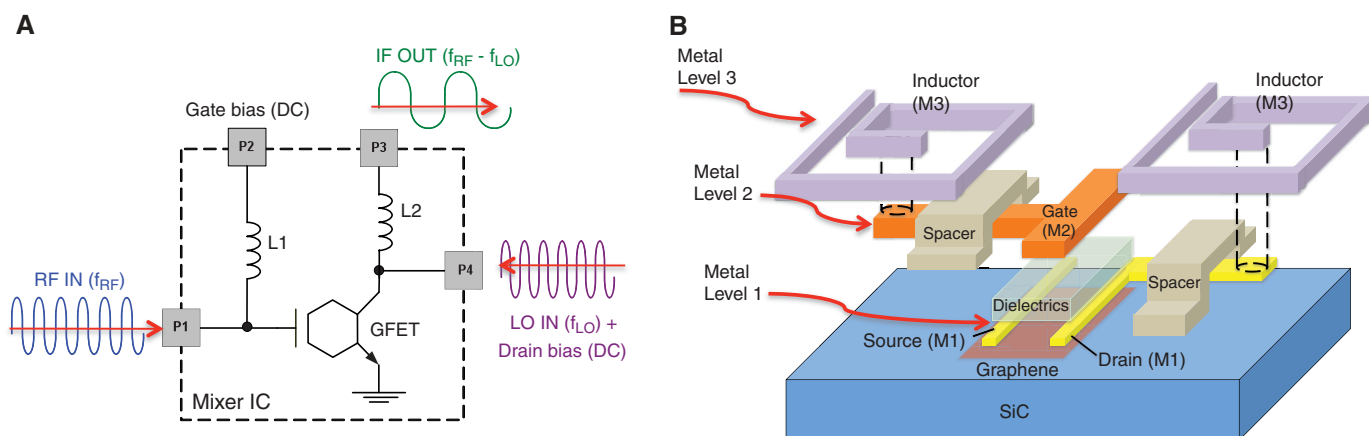
and radio-frequency (RF) applications (1–4) because of its high carrier mobility and saturation velocity (5). The planar structure and the feasi-

bility of large-area graphene synthesis facilitate the adoption of top-down device fabrication techniques. Graphene transistors with intrinsic cut-off frequencies beyond 100 GHz have been recently achieved by several groups using graphene films synthesized by various methods, including epitaxial growth on SiC (6, 7), chemical vapor deposition (CVD) on Cu (8), and mechanical exfoliation (9, 10). The monolithic integration of transistors with interconnects and other components is an essential requirement for any semiconductor material to achieve a widespread technological impact. Previous attempts to make circuits based on graphene have used an indi-

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**Fig. 1. (A)** Circuit diagram of a four-port graphene RF frequency mixer. The scope of the graphene IC is confined by the dashed box. The hexagonal shape represents a graphene FET. **(B)** Schematic exploded illustration of a graphene mixer circuit. The critical design aspects include a top-gated graphene tran-

sistor and two inductors connected to the gate and the drain of the GFET. Three distinct metals layers of the graphene IC are represented by M1, M2, and M3. A layer of 120-nm-thick SiO<sub>2</sub> is used as the isolation spacer to electrically separate the inductors (M3) from the underlying interconnects (M1 and M2).



vidual graphene transistor connected to external passive elements (11–13). Such heterogeneous circuitry inevitably results in degraded performance dominated by interconnects and parasitics rather than the intrinsic properties of graphene device. For example, Wang *et al.* demonstrated an RF frequency mixer operating at a few tens of megahertz based on a single graphene transistor (12).

Despite recent progress in graphene synthesis and device performance, scalable integration of graphene into a practical circuit remains challenging. The key difficulties stem from the distinct materials properties of graphene with respect to those of conventional semiconductors, such as a different ohmic contact formation mechanism (14), poor adhesion with metals and oxides (15, 16), and its vulnerability to damage in plasma processing. Thus, bridging the technological gap between a single device and a practical graphene circuit on the wafer scale requires innovative integration processes and circuit designs. Here, we describe wafer-scalable processes that have been developed to fabricate arrays of graphene analog circuits, each consisting of one graphene transistor and two inductors, all compactly integrated on a single SiC substrate. The entire integrated circuit (IC), including the contact pads, is less than 1 mm<sup>2</sup>,

and successful integration is verified by operating it as an RF mixer at a designated gigahertz frequency range.

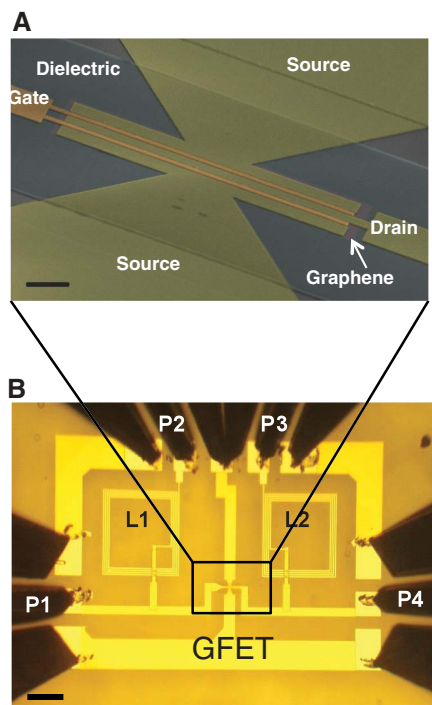
The previously demonstrated frequency multipliers and mixers using graphene field-effect transistors (GFETs) were based on the ambipolar transport characteristics of CVD and exfoliated graphene (11–13). The mixer circuit design exploits a general gate-driven and drain-driven current modulation behavior in GFETs that can be used in both ambipolar and unipolar devices. Mixers are electrical circuits used for frequency conversion and are critical components in modern RF communication systems. Two high-frequency signals, an RF signal at a frequency  $f_{\text{RF}}$  and a local oscillator (LO) signal at a frequency  $f_{\text{LO}}$ , are

applied to the gate and the drain of the GFET through port P1 and port P4, respectively (Fig. 1A). The graphene transistor is modulated by both signals and produces a drain current that contains the mixed frequencies, the sum ( $f_{\text{RF}} + f_{\text{LO}}$ ), and the difference ( $f_{\text{RF}} - f_{\text{LO}}$ ), the intermediate frequency ( $f_{\text{IF}}$ ) of the input frequencies. The integrated inductors complement the graphene FET to form an integrated RF mixer. Inductor L1 resonates out the parasitic capacitances from the input RF pad and the gate of the graphene FET, while inductor L2 provides an input match to the LO signal and acts as a low-pass filter between the drain of the FET and the output port P3. In practice (e.g., in a radio receiver application), frequencies of the RF and LO input signals differ by only a small amount, and the output signal component of interest is  $f_{\text{IF}}$ .

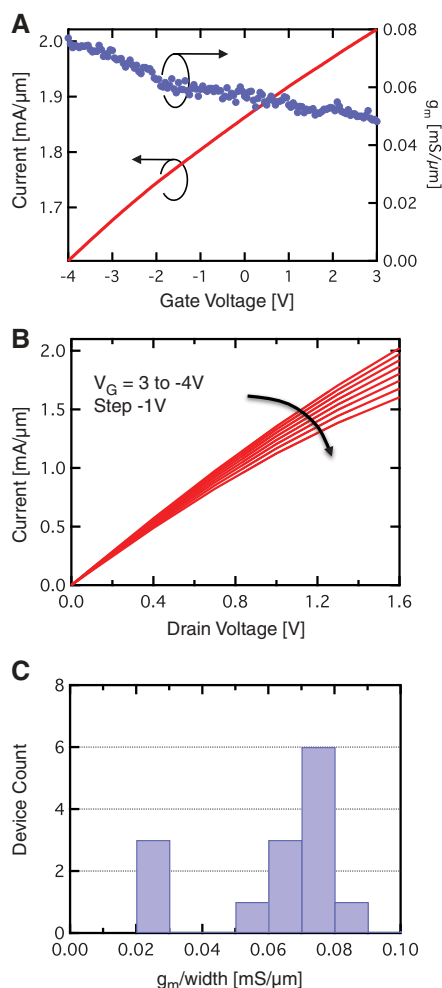
Graphene circuits were fabricated on a semi-insulating SiC wafer. A two- or three-layer graphene film was epitaxially grown on the Si face of the SiC substrate at temperatures above 1400°C (17–19), as confirmed by Raman spectroscopy and optical absorption measurements (see fig. S1 in the supporting online material). Fabrication of the graphene IC began with top-gated, two-finger graphene FETs (Fig. 2A), followed by integration with on-chip inductors. To form the active channel of the transistor, we spin-coated the graphene-SiC wafer with a layer of 140-nm-thick PMMA [poly(methyl methacrylate)] followed by a layer of 20-nm-thick HSQ (hydrogen silsesquioxane). The FET channel was defined by e-beam lithography (EBL); the surrounding graphene was removed by an oxygen plasma with the exposed HSQ film as the protecting mask. The HSQ-PMMA stack over the channel region was subsequently removed by acetone.

The removal of graphene film on SiC outside of the active channel region was critical to achieve good adhesion of thick metals in the subsequent deposition processes. The ohmic source and drain contacts, contact pads, and gate electrode were all made of the same metal stack of 20-nm Pd, 40-nm Au. The gate length was 550 nm and the distance between source and drain contacts was 600 nm. The gate dielectric was deposited by evaporating two 2-nm layers of Al metal onto the graphene channel that were then oxidized at elevated temperatures (~120°C) in air to form a seed layer for the subsequent deposition of Al<sub>2</sub>O<sub>3</sub> (20 nm) by atomic layer deposition (14). The capacitance of the resulting gate dielectric stack was ~2.5 × 10<sup>-7</sup> F/cm<sup>2</sup>.

Inductors were defined by EBL and formed by depositing 1-μm-thick Al metal. A layer of 120-nm-thick SiO<sub>2</sub>, deposited by e-beam evaporation, was used to isolate the inductor loops from the underlying metal interconnects. The inductor had a value of 5.2 nH, a self-resonant frequency of ~10 GHz, and quality factor ( $Q$ ) of 5, as measured on a stand-alone test site. The inductance was designed to achieve a target operation frequency of 5 GHz for the mixer circuit, and the quality factor of 5 is appropriate for broadband



**Fig. 2.** Images of graphene ICs. **(A)** Scanning electron image of a top-gated, dual-channel graphene transistor used in the mixer IC. The gate length is 550 nm and the total channel width, including both channels, is 30 μm. Scale bar, 2 μm. **(B)** Optical image of a completed graphene mixer including contact pads. The ground-signal-ground configuration is implemented for the probe pads suitable for direct RF testing. Scale bar, 100 μm.



**Fig. 3.** **(A)** The drain current  $I_d$  of a 550-nm-gate-length graphene FET as a function of gate voltage  $V_g$  at a drain bias of 1.6 V with the source electrode grounded. The current shown was normalized with respect to the total channel width. The device conductance  $g_m$  is shown on the right axis. **(B)** The measured drain current  $I_d$  as a function of drain bias of the graphene FET for various top-gate voltages. **(C)** Distribution of peak  $g_m$  of graphene FETs, all of the same gate length of 550 nm and fabricated on the same wafer.

operation. Figure 2B shows an image of the completed graphene circuit, including the inductors, GFET, and contact pads. The layout of the entire die, containing arrays of graphene ICs used as monitor devices and other testing components, is shown in fig. S3.

Figure 3A shows the current  $I_d$  and transconductance  $g_m$  as a function of gate voltage  $V_g$  of a typical graphene transistor with a gate length of 550 nm at drain voltage  $V_d = 1.6$  V measured at room temperature. Within the gate voltage range studied here, the GFETs always exhibited dominant n-type transport (6, 17, 19), which differs from the ambipolar characteristics typically observed in CVD and exfoliated graphene. The output characteristics of the GFET (Fig. 3B) exhibited a nearly linear  $I_d$ - $V_d$  dependence for all gate voltages up to a drain voltage of 1.6 V. These triode-like output characteristics resulted in a device transconductance that increased with rising  $V_d$ . At a drain bias of 1.6 V, a current density above 2 mA/ $\mu\text{m}$  and a transconductance of 80  $\mu\text{S}/\mu\text{m}$  were achieved. The measured current density was enhanced by the high intrinsic doping level (up to  $10^{13} \text{ cm}^{-2}$ ) of graphene. Figure 3C shows the distribution of the peak transconductance of 13 GFETs fabricated on the same wafer, with most devices ranging between 60 and 80  $\mu\text{S}/\mu\text{m}$ . The contact resistance of the graphene transistor, which included the source and drain contacts, was about 600  $\text{ohm}\cdot\mu\text{m}$ . On the basis of the measured dc transconductance and gate capacitance, the intrinsic cut-off frequency of the GFET is estimated to be  $\sim 9$  GHz for a gate length of 550 nm and drain bias of 1.6 V.

Unlike conventional mixers that are typically realized by applying LO and RF signals to a nonlinear device, such as a Schottky-barrier diode, the mixer IC discussed here uses channel resistance modulation of the graphene FET to achieve frequency mixing. The drain current exhibits a nearly linear dependence with respect to gate and drain voltages (Fig. 3, A and B) and can be expressed, to the first-order approximation, by  $I_d \approx A \cdot (B + g_m \cdot V_g) \cdot V_d$ , where  $A$  and  $B$  are constants. The frequency mixing becomes evident in view of the product term of  $V_g$  and  $V_d$ . Because the output signal is proportional to the drain current modulation, the power of output signal  $f_{IF}$  is proportional to the product of the transconductance  $g_m = \left(\frac{dI_d}{dV_g}\right)$  and the output conductance  $g_d = \left(\frac{dI_d}{dV_d}\right)$ . Although current saturation in the  $I_d$ - $V_d$  curves, which corresponding to a small  $g_d$ , is a desirable feature for RF and analog applications in general, it remains challenging to achieve well-behaved current saturation in graphene FETs, especially for small gate lengths (20, 21). Most graphene devices exhibit a triode-like  $I_d$ - $V_d$  behavior such as the one shown in Fig. 3B. By using the finite conductance  $g_d$  of graphene transistors to realize mixing, the output signal is linearly proportional to both gate and drain input signals. This configuration has been

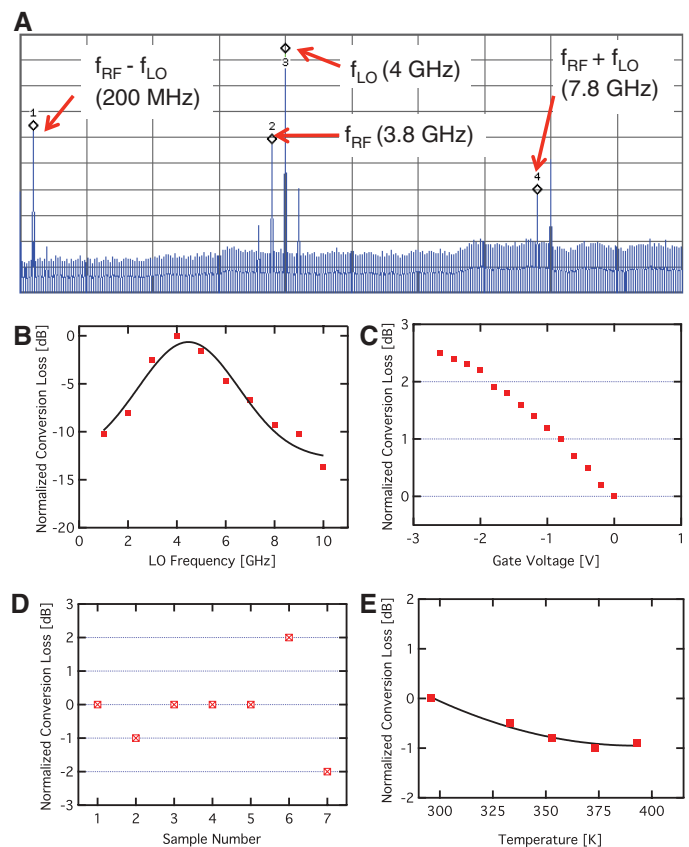
previously used with GaAs FETs operating in the linear region (22), achieving a superior linearity compared to mixers that use diodes or FETs operating in saturation mode.

Figure 4A displays the output frequency spectrum of the graphene mixer with input signals  $f_{RF} = 3.8$  GHz and  $f_{LO} = 4$  GHz and a drain bias of 2 V. The frequency mixing function was visible as two tones observed at frequencies  $f_{IF}$  of 200 MHz and  $f_{RF} + f_{LO}$  of 7.8 GHz. The higher-frequency tone was attenuated by the drain inductor and hence showed lower amplitude. The power of signal  $f_{IF}$  was proportional to that of the input RF signal up to  $P_{RF} = 12$  dBm for an LO signal as high as  $P_{LO} = 20$  dBm (fig. S5); this high level of linearity is expected from the mixer configuration as described above. The conversion loss, defined as the power ratio of the IF signal  $P_{IF}$  and the input RF signal  $P_{RF}$ , is about  $-27$  dB. Figure 4B shows the dependence of conversion loss of the mixer on the LO frequency while maintaining  $f_{IF}$  at 200 MHz. The performance of this graphene mixer peaked around  $f_{LO} \sim 4.5$  GHz, which is in good agreement with the target frequency of 5 GHz. These results not only validate the proper function of the graphene circuit as an RF mixer, but also demonstrate successful inte-

gration and operation of an active graphene device coupled with supporting components on a single chip.

Figure 4C shows the dependence of conversion loss of the mixer on dc gate voltage, revealing a trend that qualitatively follows the variation of transconductance  $g_m$  as a function of  $V_g$  (Fig. 3A). The strong correlation between the conversion loss and  $g_m$  shows that performance of the graphene mixer was determined by the properties of GFET itself and not by parasitic signal transmission. The conversion gain can be enhanced by further improving  $g_m$ , e.g., by adopting a thinner oxide and a shorter channel length. As the drain voltage increases from 0.3 to 2 V, the conversion loss is improved by about +3 dB, in agreement with the increasing  $g_m$  with  $V_d$  (Fig. 3B). The conversion loss of all mixers fabricated on the same wafer and measured at identical dc bias conditions varied within  $\pm 2$  dB (Fig. 4D). To evaluate temperature response of these graphene ICs, we measured the mixer performance as a function of temperature up to  $T = 400$  K. In conventional semiconductor devices, the performance is usually severely degraded as  $T$  increases, and additional feedback circuitry is required to minimize the thermal sensitivity. In contrast, the con-

**Fig. 4. (A)** A snapshot of output spectrum, between 0 and 10 GHz, of the mixer taken from the spectrum analyzer with  $f_{RF} = 3.8$  GHz and  $f_{LO} = 4$  GHz. Each x and y division corresponds to 1 GHz and 10 dBm, respectively. The graphene FET was biased at a drain bias of 2 V and a gate voltage of  $-2$  V. The input RF power was adjusted to 0 dBm, so that the output spectrum power measured the actual loss (gain) with respect to the RF input. The frequency mixing was visible with two peaks observed at frequencies of 200 MHz and 7.8 GHz with signal power of  $-27$  and  $-52$  dBm, respectively. **(B)** Measured conversion loss of the graphene frequency mixer as a function of LO frequency. The conversion loss was normalized to the value at  $f_{LO} = 4$  GHz. The solid line is a guide to the eye. **(C)** Conversion loss of the mixer measured as a function of  $V_g$  of the graphene FET, normalized to the value at  $V_g = -3$  V. **(D)** Distribution of the conversion loss of seven working graphene mixer ICs, all fabricated on the same wafer. The conversion loss was normalized to their average value. **(E)** Temperature dependence of conversion loss of the graphene mixer between 300 and 400 K measured at  $V_d = 1$  V and  $V_g = -1$  V. The conversion loss was normalized to the value at 300 K, showing performance degradation less than 1 dB in this temperature range. The solid line is a guide to the eye.





version loss of the graphene mixer exhibits little, if any, temperature dependence with a variation below 1 dB in this wide range from 300 to 400 K (Fig. 4E). The absence of strong temperature dependence of graphene ICs is attributed to a degenerate doping level in these GFETs and a nearly  $T$ -independent scattering mechanism associated with optical phonons at high biases (8, 20, 23).

The integration techniques and operating principles of graphene circuits described here can be applied to CVD graphene and are also compatible with optical lithography for reduced cost and enhanced throughput. The fabricated graphene IC achieved a 27-dB conversion loss at 4 GHz. In comparison, previously reported graphene-based mixers operated at 10 MHz with a 40-dB conversion loss (14), and a commercially available GaAs-based mixer achieves 7-dB conversion loss at 1.95 GHz (24). In addition, the operation of these mixers required external passive components, whereas the graphene mixer presented here was integrated. The use of thin high- $k$  dielectrics (e.g., 2 nm  $\text{HfO}_2$ ) or highly scaled gate lengths (e.g., 40 nm) (8) could improve the FET transconductance by a factor of 10, which would lead to an enhancement of more than 20 dB in conversion gain for the graphene mixer. Moreover,

such FET improvement in combination with the integration scheme described here would also enable other graphene-based circuits, such as amplifiers and oscillators, and their use in wireless communication systems.

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#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/332/6035/1294/DC1](http://www.sciencemag.org/cgi/content/full/332/6035/1294/DC1)

Materials and Methods

Figs. S1 to S6

References

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# Endotoxin-Induced Structural Transformations in Liquid Crystalline Droplets

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The ordering of liquid crystals (LCs) is known to be influenced by surfaces and contaminants. Here, we report that picogram per milliliter concentrations of endotoxin in water trigger ordering transitions in micrometer-size LC droplets. The ordering transitions, which occur at surface concentrations of endotoxin that are less than  $10^{-5}$  Langmuir, are not due to adsorbate-induced changes in the interfacial energy of the LC. The sensitivity of the LC to endotoxin was measured to change by six orders of magnitude with the geometry of the LC (droplet versus slab), supporting the hypothesis that interactions of endotoxin with topological defects in the LC mediate the response of the droplets. The LC ordering transitions depend strongly on glycopospholipid structure and provide new designs for responsive soft matter.

The functional properties of inorganic and organic materials have been manipulated through the deliberate introduction of defects and grain boundaries, as well as through the partitioning of low concentrations of dopant spe-

cies to these localized regions of the materials (1–4). For soft materials, such as liquid crystals (LCs), geometrical confinement within micrometer-sized systems has been shown to lead to the formation of a range of thermodynamically stable defects with nanoscopic dimensions and varied topologies (points, lines, rings) (5–11). In this paper, we report that confinement of LCs within micrometer-sized droplets dispersed in water can lead to ordering transitions in the LC droplets that are highly specific and sensitive to a particular bacterial glycopospholipid. These ordering transitions occur at concentrations of lipid that are six orders of magnitude lower than previously reported adsorbate-driven ordering transitions in LC systems (10–13).

Endotoxin is a bacterial lipopolysaccharide comprised of a glycopospholipid (called lipid A) (Fig. 1A) in addition to two polysaccharide domains. Lipid A has six tails, and thus it is structurally distinct from all other lipids (14). We investigated the interactions of endotoxin from *Escherichia coli*, the lipid A portion of endotoxin, and the other phospholipids and surfactants shown in Fig. 1, B to D, with micrometer-sized droplets of nematic LC [4'-pentyl-4-cyanobiphenyl (5CB)] (Fig. 1E) that were dispersed initially in endotoxin-free water (15). In the absence of endotoxin, bright-field (Fig. 1F) and polarized light micrographs (Fig. 1G) revealed the presence of two point defects (surface defects, called boojums) located at the "poles" of the LC droplets, corresponding to a so-called bipolar configuration of the droplet where the LC assumes a tangential orientation at the droplet surface (Fig. 1H) (5). After the addition of endotoxin to the water at a concentration of 1  $\mu\text{g}/\text{ml}$ , both bright-field (Fig. 1I) and polarized light imaging (Fig. 1J) revealed a reordering of the LC within the droplet to a so-called radial configuration, corresponding to a single defect located at the droplet center and LC oriented perpendicular to the droplet surface (Fig. 1K) (5). Upon measuring the fraction of LC droplets within a solution of endotoxin that exhibited the radial configuration (Fig. 1L), we found that remarkably low concentrations of endotoxin (<1  $\text{pg}/\text{ml}$ ) trigger the ordering transition. Consistent with our conclusion that the interaction of the LC droplets and endotoxin was responsible for the ordering transition, the fraction of LC droplets in the radial configuration increased with the concentration of endotoxin (Fig. 1L). The response of the LC

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droplets to endotoxin was dependent on the total number of LC droplets in solution, allowing the ordering transition to be tuned over a range of endotoxin concentrations (e.g., 1 to 10 pg/ml or 1 to 100 pg/ml) (Fig. 1L). The lipid A portion of endotoxin caused similar ordering transitions in the LC droplets.

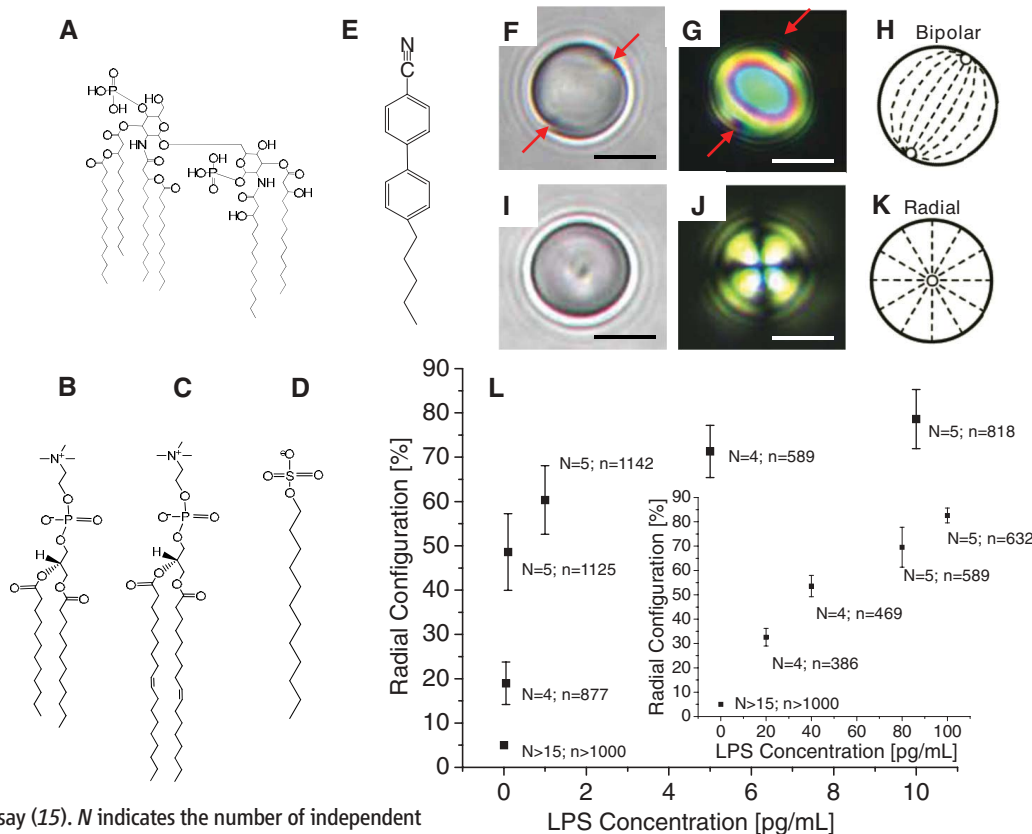
The simplest continuum description of the ordering of LCs within droplets considers the free energy,  $F$ , of each droplet to be influenced by an orientation-dependent surface free energy and an elastic strain free energy, namely  $F \approx \int W dA + \frac{K}{2} \int (\nabla n)^2 dV$ , where  $W$  is the so-called surface anchoring energy per unit area,  $A$  is the surface area of the droplet,  $K$  is the elastic modulus for strain of the LC (16),  $n$  is the director of the LC (16), and  $V$  is the volume of the LC droplet (5, 8, 10, 11). Past studies have demonstrated that lipids and synthetic surfactants can induce ordering transitions at aqueous-LC interfaces through a mechanism that involves an adsorbate-induced change in  $W$  (12, 13, 17). However, to change  $W$  to induce an ordering transition requires near saturation coverage of the interface by the adsorbate (0.1 to 1 Langmuir), typically corresponding to solution concentrations of lipids of at least  $\sim 10 \mu\text{g/ml}$  (10–13, 18). Consistent with a mechanism involving changes in  $W$ , we observed that double-tailed lipids such as 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) (Fig. 1, B and C) and single-tailed synthetic surfac-

tants such as sodium dodecylsulfate (SDS) (Fig. 1D) induced ordering transitions in the LC droplets only at concentrations greater than  $10 \mu\text{g/ml}$  (Fig. 2A). These results contrast to lipid A where ordering transitions are induced in the LC droplets at concentrations of lipid A that are at least six orders of magnitude lower (picograms per milliliter). We calculated that if all of the endotoxin in  $40 \mu\text{L}$  of a  $1 \text{ pg/ml}$  solution adsorbed uniformly over the aqueous-LC interface of  $\sim 10^4$  LC droplets (radius  $3 \mu\text{m}$ ) in one of our experiments (Fig. 1L), the interfacial density of endotoxin would be  $\sim 1 \text{ molecule}/10^6 \text{ nm}^2$  or  $\sim 10^{-5}$  of saturation monolayer coverage ( $\sim 10^{-5}$  Langmuir) (19). In contrast, the double-tailed lipids mentioned above (Fig. 1, B and C) trigger ordering transitions at interfacial concentrations that correspond to  $\sim 1 \text{ molecule}/0.6 \text{ nm}^2$  (saturation coverage is  $\sim 1 \text{ molecule}/0.4 \text{ nm}^2$ ). This pronounced difference in surface density of lipid (5 to 6 orders of magnitude) required to trigger ordering transitions within LC droplets led us to propose that, at pg/ml concentrations, endotoxin and lipid A do not trigger ordering transitions in the LC droplets through changes in  $W$  (as is the case for DLPC and DOPC) but instead trigger the ordering transitions through interaction with localized regions of the LC droplets. We hypothesized that the local regions of interaction were defined by the defects in the LC (Fig. 1, H and K).

Past studies have established that nematic LCs, when confined in certain geometries, cannot satisfy the boundary conditions of the system

through continuous strain (splay, bend, and twist) of the LC (5, 7, 8, 10, 11). Instead, the LC satisfies the boundary conditions through the generation of nanoscopic regions within which the molecules comprising the LC possess low levels of orientational order, namely, defects such as the point defects shown in Fig. 1, H and K. We tested the role of defects in the LC ordering transitions induced by endotoxin and lipid A by changing the geometry of the LC system. First, we measured ordering transitions induced by spontaneous adsorption of endotoxin or lipid A at planar interfaces of LC that lack the defects generated by the spherical geometry of the droplets. At these planar interfaces, neither lipid A nor endotoxin triggered an ordering transition in the LC until the concentration exceeded  $\sim 1 \mu\text{g/ml}$  (fig. S1) (12, 15, 20, 21). Second, we formed Langmuir monolayers of lipid A of known density at the surface of water and transferred the lipid A monolayers onto planar aqueous-LC interfaces using the Langmuir-Schaefer transfer method (as illustrated in Fig. 2B) (13, 15). These measurements revealed that a surface concentration of lipid A of  $\sim 1 \text{ molecule}/1.15 \text{ nm}^2$  was required to cause an ordering transition at the planar LC interface (Fig. 2C), a surface concentration that is six orders of magnitude greater than the surface concentration driving the ordering transition of the LC droplets. These observations, when combined with the results reported above, support our hypothesis that endotoxin and lipid A trigger or-

**Fig. 1.** (A) Lipid A portion of endotoxin. (B) DLPC. (C) DOPC. (D) SDS. (E) 5CB. (F and G) Bright-field (F) and polarized light (G, crossed-polars) micrographs of an LC droplet in endotoxin-free water (15). The red arrows indicate boojums at the aqueous-LC interface of the droplet. (H) Schematic illustration of the bipolar configuration of the LC droplet corresponding to (F) and (G). (I and J) Bright-field (I) and polarized light (J, crossed-polars) micrographs of an LC droplet after exposure to endotoxin from *E. coli* (O127:B8;  $1 \mu\text{g/ml}$ ) in water. (K) Schematic illustration of the radial configuration of the LC droplet corresponding to (I) and (J). (L) Percentage of 8300 LC droplets in  $40 \mu\text{L}$  of water that exhibited a radial configuration, plotted as a function of endotoxin concentration. The inset shows the response of the LC droplets when 94,000 droplets were used. The LC-in-water emulsion droplets (diameters of  $\sim 4$  to  $8 \mu\text{m}$ ) were prepared by sonication of nematic 5CB in water that was free of endotoxin (15). The droplet numbers were determined using flow cytometry (15). Endotoxin concentrations were validated using an independently performed Limulus amoebocyte lysate assay (15).  $N$  indicates the number of independent experiments, and  $n$  indicates the total number of LC emulsion droplets that were analyzed. Scale bars,  $5 \mu\text{m}$ .



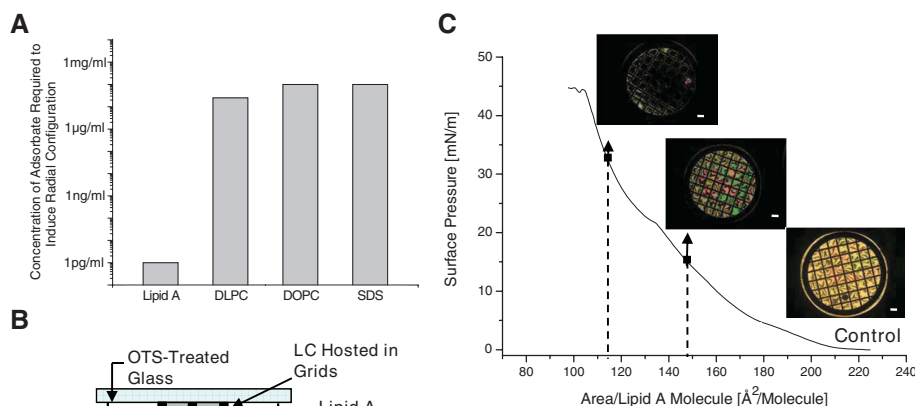


dering transitions in micrometer-sized LC droplets through interaction with defects in the LC that are induced by the spherical geometry of the LC droplets.

To provide further insight into the interaction of endotoxin with the defects of the LC droplets, we performed confocal fluorescence microscopy using boron dipyrromethene (BODIPY)-labeled

endotoxin (Fig. 3A) (15). These measurements confirmed that endotoxin was localized at the point defect formed at the center of each LC droplet with a radial configuration. Interestingly, after deliberate photobleaching of the BODIPY-labeled endotoxin at the point defect, we measured a time-dependent recovery of the fluorescence (Fig. 3B), indicating an exchange of lipid between the cen-

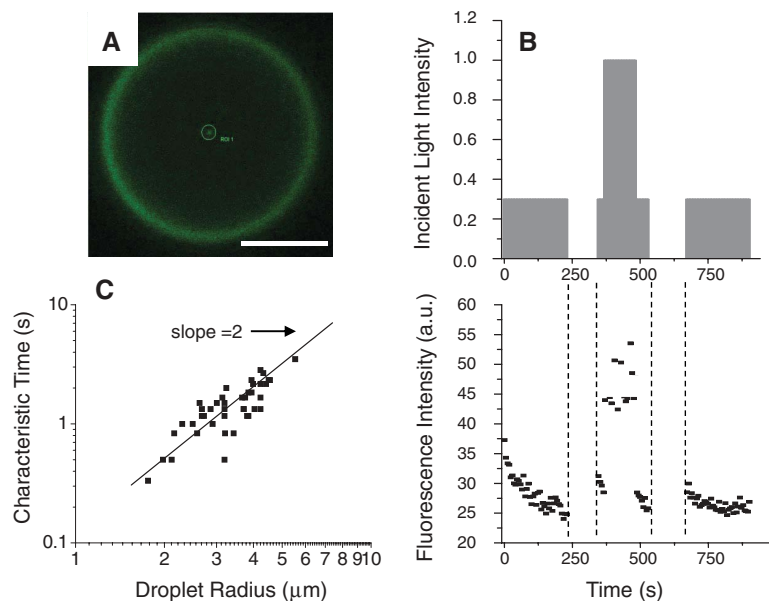
ter and surface of the droplet that occurred over tens of seconds. We also heated an emulsion of 5CB free of endotoxin above the clearing temperature of the nematic phase ( $T_{\text{iso}} = 35^\circ\text{C}$ ; experiments were performed at  $50^\circ\text{C}$ ), added 10 pg/mL of endotoxin, and then cooled the sample through the isotropic-to-nematic phase transition. We measured the characteristic time interval between the formation of the nematic phase during cooling and the establishment of radial ordering (Fig. 3C) (22, 23). The time interval was found to scale with the square of the droplet radius, consistent with a physical process underlying the ordering transition that involved diffusion of the lipid. We calculated the time for diffusion of lipid across the surface of the LC droplet (calculated as  $t_1 = L_A^2/4D_s$ , where  $L_A$  corresponds to  $\pi R/2$ , the distance along the surface from the equator to the pole) to be in good agreement with the data in Fig. 3C [using  $D_s \sim 10 \times 10^{-12} \text{ m}^2/\text{s}$  (12)]. The surface diffusion time for a droplet with a radius of 3  $\mu\text{m}$  was calculated to be  $\sim 0.6 \text{ s}$ ; the experimentally measured characteristic time was  $\sim 1 \text{ s}$  (Fig. 3C) (22, 23). This result suggests a physical process in which the dynamics of the ordering transition induced by endotoxin are governed by the lateral transport of endotoxin across the surface of the LC droplet, a process that would be necessary to localize endotoxin at surface defects (boojums) to initiate the ordering transition. We note that past studies have reported observations of the localization of inorganic colloids at defects generated in LC systems (24, 25), including stabilization of blue phases (26). We also observed 10 pg/mL of endotoxin to trigger the ordering transition from the bipolar to the radial configuration through a kinetic pathway that was distinct from that observed during the surface-driven ordering transitions caused by DLPC, SDS, lecithins and concentrations of endotoxin (50  $\mu\text{g}/\text{mL}$ ) that were sufficient to saturate the LC interface (10, 11, 27). The distinct kinetic pathway triggered by 10 pg/mL of



**Fig. 2.** (A) Bulk concentrations of lipids or surfactants in solution (40  $\mu\text{L}$ ) required to cause at least 50% of 8300 LC droplets added to each solution to adopt a radial configuration. (B) Schematic illustration of the experimental apparatus used to transfer Langmuir monolayers

of lipid A at prescribed surface densities from an air-water interface onto a planar aqueous-LC interface (13). In brief, micrometer-thick films of nematic 5CB (with planar interfaces) were prepared within the pores of Au grids supported on glass slides treated with octadecyltrichlorosilane (to cause perpendicular ordering of the LC). The supported LC was then immersed downward through the lipid A monolayer at the surface of the water (15) to transfer the monolayer onto the planar aqueous-LC interface. (C) Surface pressure-area isotherm measured for a Langmuir monolayer of lipid A and the optical appearance (between crossed polars) of films of 5CB with planar interfaces (hosted within the metallic grids) after transfer of Langmuir films of lipid A at the indicated interfacial density onto the 5CB-aqueous interface [see (B) for details]. The control corresponds to LC passed into endotoxin-free water. The bright optical appearance of the control indicates an orientation of the LC that is parallel to the aqueous-LC interface. The optical appearance of the sample prepared at an area per molecule of lipid A of  $148 \text{ \AA}^2/\text{molecule}$  indicates a tilted state of the LC. The dark optical appearance of the sample prepared at  $115 \text{ \AA}^2/\text{molecule}$  indicates a perpendicular orientation of the LC at the aqueous interface. Scale bars, 300  $\mu\text{m}$ .

**Fig. 3.** (A) Confocal fluorescent micrograph of an LC droplet in contact with a solution of BODIPY-labeled endotoxin. The point defect at the center of the droplet with a radial configuration exhibits a strong fluorescence signal. The concentration of endotoxin in solution was 20  $\mu\text{g}/\text{mL}$  in order to achieve a sufficient signal intensity to image the droplets. Imaging was performed using an argon laser at an excitation wavelength of 488, and the detector collected the emission with wavelengths from 499 nm to 634 nm (15). Scale bar, 5  $\mu\text{m}$ . (B) Photobleaching of the BODIPY-labeled endotoxin at the center of the LC droplet. The upper panel shows the intensity of the excitation laser, and the lower panel shows the corresponding fluorescence intensity. Loss of fluorescence signal is apparent during the periods of sample illumination (e.g., 0 to 230 s), and recovery of fluorescence is apparent in the periods of time when the laser incident on the sample was blocked (e.g., 230 to 350 s); A.U. indicates arbitrary units. The incident light intensity is indicated as a fraction of the maximum laser power. (C) Time taken for LC droplets to exhibit radial ordering after a thermal quench from the isotropic to nematic phase, plotted as a function of the droplet radius. The experiment was performed with 21,500 LC droplets dispersed in 100  $\mu\text{L}$  of solution containing 10 pg/mL endotoxin. The line shown in the figure has a slope of 2.



endotoxin provides further support for our conclusion that the ordering transitions at low concentrations of endotoxin are not caused by changes in surface anchoring energy ( $W$ ).

In summary, our experiments establish that the effect of endotoxin at concentrations of  $\sim 1$  pg/ml on the surface anchoring energy of LCs is negligible; ordering transitions in LC droplets driven by changes in surface anchoring energy require six orders of magnitude higher surface concentrations of lipid than are present in our experiments with endotoxin. Furthermore, we do not expect the endotoxin to change the elastic moduli of the LC and thus drive the ordering transition through a lowering of the elastic energy. Instead, the experimental results reported in this paper are consistent with the proposition that the ordering transitions in micrometer-sized LC droplets are driven by the interactions of endotoxin with topological defects induced by the geometry of the LC droplets. Our observation that the ordering transitions of the LC droplets are specific to the lipid structure of endotoxin suggests that endotoxin may be forming organized assemblies within the cores of the defects to change the defect energies to favor stabilization of the radial configuration over the bipolar structure. In comparison with previously reported ordering transitions involving planar LC films, we find that endotoxin-driven ordering transitions in LC droplets are  $\sim 10^6$  times more sensitive. This sensitivity and structure-based selectivity of ordering transitions in LC droplets suggests new principles for the design of responsive LC systems, particularly for the design of sensors that respond to targeted biological analytes (28, 29).

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28. Endotoxin is an indicator of bacterial contamination. Detection of endotoxin in purified water (in the 1 to 100 pg/ml range) is widely performed during manufacturing processes for pharmaceutical products (including validation of water used in such processes) and biomedical devices, in a variety of public health contexts (particularly analysis of aerosols in industrial and indoor environments) and for biological research.
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## Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1195639/DC1  
Materials and Methods  
Figs. S1 to S4  
References

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# Methylhydroxycarbene: Tunneling Control of a Chemical Reaction

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Chemical reactivity is conventionally understood in broad terms of kinetic versus thermodynamic control, wherein the decisive factor is the lowest activation barrier among the various reaction paths or the lowest free energy of the final products, respectively. We demonstrate that quantum-mechanical tunneling can supersede traditional kinetic control and direct a reaction exclusively to a product whose reaction path has a higher barrier. Specifically, we prepared methylhydroxycarbene ( $\text{H}_3\text{C}-\text{C}-\text{OH}$ ) via vacuum pyrolysis of pyruvic acid at about 1200 kelvin (K), followed by argon matrix trapping at 11 K. The previously elusive carbene, characterized by ultraviolet and infrared spectroscopy as well as exacting quantum-mechanical computations, undergoes a facile [1,2]hydrogen shift to acetaldehyde via tunneling under a barrier of 28.0 kilocalories per mole ( $\text{kcal mol}^{-1}$ ), with a half-life of around 1 hour. The analogous isomerization to vinyl alcohol has a substantially lower barrier of 22.6  $\text{kcal mol}^{-1}$  but is precluded at low temperature by the greater width of the potential energy profile for tunneling.

The conceptual foundations of chemical kinetics were laid by the development of transition state theory (TST) (1, 2), a powerful model that describes a chemical reaction as the passage of a system through an activated complex and over the top of a barrier on a molec-

ular potential energy surface (3). This passage entails the evolution of the atomic positions from reactants to products along the most favorable path, whose critical point of highest energy is the transition state, a saddle point on the overall potential energy landscape.

The practical application of such theories of physical chemistry to competing organic reactions led to the principle of kinetic versus thermodynamic control of chemical transformations, first proposed graphically by Woodward and Baer (4) and then explicitly by Ingold and co-workers (5). The advent of multistep organic syntheses of complex molecules helped establish this principle as an effective means of predicting chemical selectivity.

As originally constructed and usually employed, kinetic control refers to a reaction system regulated by the relative energies of competing transition states, the process with the lower activation barrier being preferred. More general but less intuitive definitions of kinetic control that do not invoke TST have been proposed (6). In reactions under thermodynamic control, the products form in proportions determined by the equilibrium constants for their interconversion, the species with the lower free energy being favored;

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there must be sufficient thermal activation in the system to surmount the barriers for those interconversions necessary for equilibration.

The rate of a reaction can be substantially affected by quantum-mechanical tunneling (7), a classically forbidden process whereby the system yields products by passing under an energy barrier rather than over the top. Even in Eyring's earliest formulation of TST, the possibility of tunneling was recognized (1). Tunneling becomes especially important at low temperatures and for reactions involving light atoms. Typical theoretical treatments of chemical kinetics are based on classical TST, with tunneling included as a secondary correction factor for reaction rates (8). Here we present a combination of experiments and theoretical analyses on methylhydroxycarbene (**1**), pyruvic acid (**2**), acetaldehyde (**3**), and vinyl alcohol (**4**) (Fig. 1) to demonstrate that tunneling can dominate a reaction mechanism sufficiently to redirect the outcome away from traditional kinetic control.

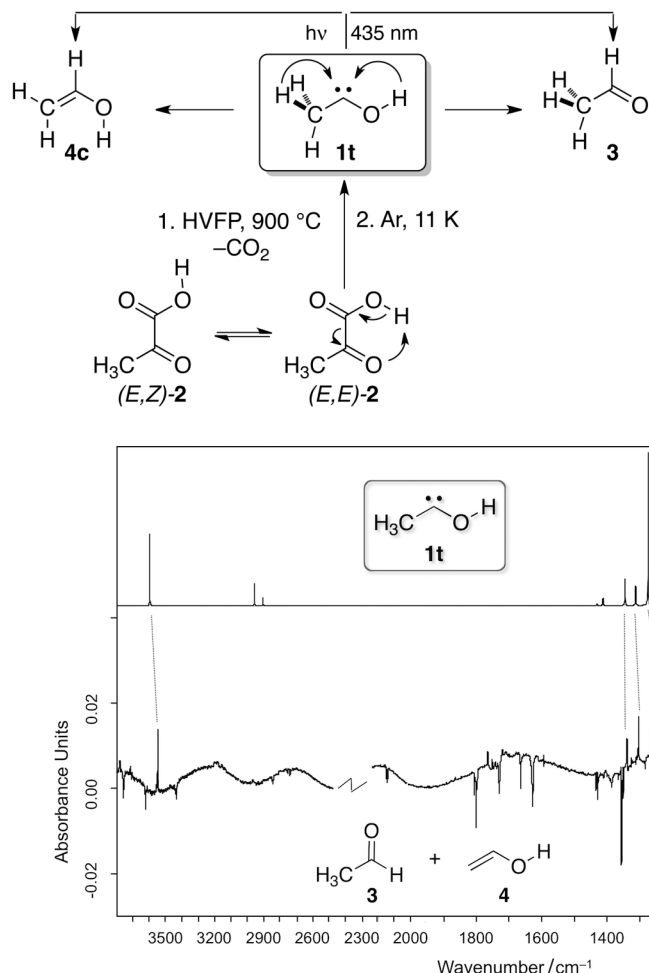
Recently, we observed (9–11) that hydroxymethylene (**5**, H–C–OH), the simplest hydroxycarbene,

exhibits remarkably fast intramolecular H tunneling (with a half-life  $t_{1/2} \approx 2$  hours) under a large barrier of nearly 30 kcal mol<sup>−1</sup>. This unexpected isomerization mechanism, yielding formaldehyde, helps explain why **5**, an implicated intermediate in coal liquefaction (Fischer-Tropsch chemistry) (12) as well as the prebiotic formation of carbohydrates (13), had not been isolated previously. Similarly, phenylhydroxycarbene (**6**, Ph–C–OH) undergoes facile H tunneling to benzaldehyde under a comparable barrier (14). Neither **5** nor **6** possesses an H atom bound to an  $\alpha$  carbon, and thus no potentially competitive [1,2]H-shift rearrangement is possible. One of the simplest structures with more than one possible channel for [1,2]H tunneling is methylhydroxycarbene (or hydroxyethylidene, **1**, Fig. 1), in which H transfer could occur from either the O–H or C–H bond. Carbene **1** is a common ligand in transition metal complexes and has also been posited as the activated form of acetaldehyde in the pyruvate cycle (15), as bound within the so-called Breslow intermediate (16).

Although **1** has not yet been characterized in isolated form, its presence has been inferred in mass spectrometric studies (17, 18). Other studies of the gas-phase pyrolysis and photolysis of pyruvic acid (**2**) (19–22), as well as the reactions of arc-generated C atoms with carbonyl compounds (23), have suggested the existence of **1** as an intermediate that rearranges to acetaldehyde (**3**) much more rapidly than to vinyl alcohol (**4**, ethenol) (19, 21–23). The preference for the formation of **3** is enigmatic and at variance with traditional kinetic control, because the computed H-shift barrier from **1** to **4** is lower than that from **1** to **3** (24). The intermediacy of **4** en route to **3** through a [1,3]H shift was excluded on the basis of deuterium labeling experiments (19, 21, 23). An unrecognized factor in these previous observations is the H-tunneling mechanism discovered here for the isomerization of **1** that overrides the expected kinetic preference for **4** and changes the course of the reaction to yield product **3** exclusively.

Building on the successful matrix isolation of hydroxycarbenes **5** and **6** as well as dihydroxycarbene (**7**) (9, 14, 25), we report here the synthesis, spectroscopic identification, and reactivity of **1**. Our approach (Fig. 1) uses the thermal extrusion of CO<sub>2</sub> from **2** under high-vacuum flash pyrolysis conditions and the subsequent trapping of the products in an Ar matrix at 11 K, as described previously (9). Several trials were performed in order to determine the optimal pyrolysis temperature (900°C). Under these conditions the decarboxylation reaction was not complete. Unreacted **2** was present in the Ar matrix in its two most stable conformers, having symmetry designations (*E,E*) and (*E,Z*) with respect to the central bonds of the (O=C–C=O, O=C–O–H) chains. The less stable (*E,Z*) form was strongly enhanced relative to the deposition without pyrolysis (19, 26). The (*E,Z*) rotamer slowly converts to the more stable (*E,E*) form through H tunneling at 11 K with a  $t_{1/2} \approx 10$  hours, as observed for other carboxylic acids (27, 28). The main pyrolysis products in the matrix were CO<sub>2</sub>, acetaldehyde (**3**), and very small amounts of vinyl alcohol (**4**) (29). The yield of matrix-isolated **1** as compared to that of **3** was estimated to be 2 to 5% through comparison of their experimental and computed infrared (IR) band intensities. Irradiation of the pyrolysis products with light at a wavelength ( $\lambda$ ) = of 435 nm led to fast and complete disappearance of the IR bands of **1** and to the concomitant enhancement of signals for **3** and **4**. By measuring the spectral differences of irradiated and non-irradiated matrices, it was possible to elaborate the vibrational and electronic properties of **1**, despite its low concentration in the complex reaction mixture. The OD isotopologue of methylhydroxycarbene (**d-1**) was prepared from CH<sub>3</sub>COCO<sub>2</sub>D (**d-2**) and characterized analogously.

The experimental IR spectra of the *s-trans* isomers **1t** and **d-1t** were assigned by comparison with computed anharmonic fundamental ( $\nu$ ) vibrational frequencies, as shown in Fig. 2 and table S7. Previous studies (9, 30) of the HCOH



**Fig. 2.** (Lower trace) IR difference spectrum of *trans*-methylhydroxycarbene (**1t**, C<sub>s</sub> point group) in an Ar matrix at 11 K; absorption differences were taken after irradiation at  $\lambda = 435$  nm for 2 min (see fig. S1 for the raw spectra). The downward peaks arise from the photorearrangement to acetaldehyde (**3**) and *s-cis*-vinyl alcohol (**4c**). Taking difference spectra is a well-established approach to identifying a species by selective photolysis at wavelengths close to the target's maximum absorption. (Upper trace) Computed AE-CCSD(T)/cc-pCVQZ anharmonic fundamental ( $\nu$ ) vibrational frequencies with corresponding AE-CCSD(T)/cc-pCVTZ intensities.

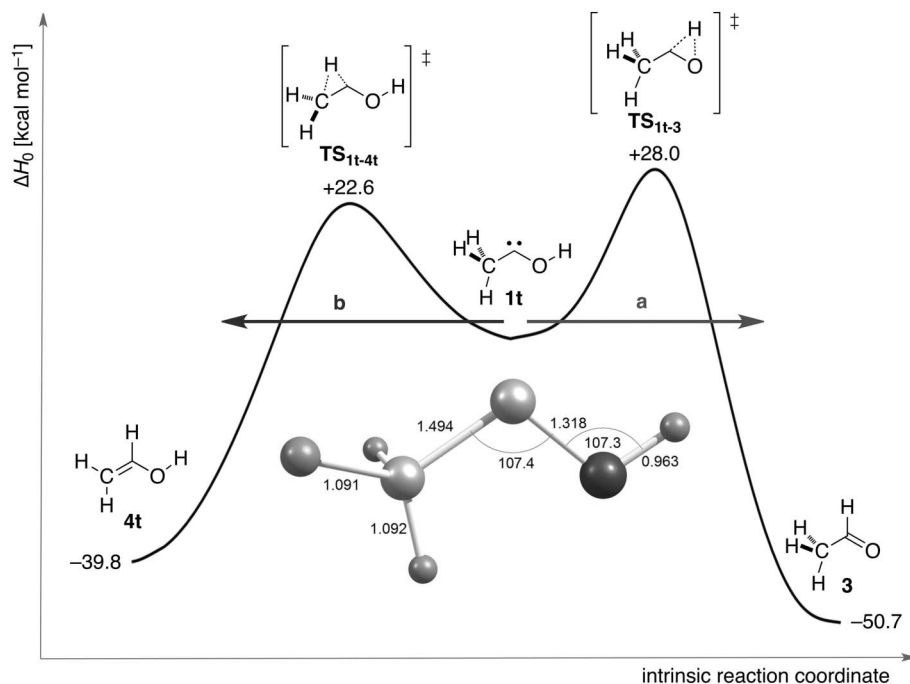
parent molecule **5** have documented the importance of vibrational anharmonicity in hydroxycarbenes. The theoretical vibrational spectra of **1t** and **d-1t** were obtained by applying second-order vibrational perturbation theory to a complete quartic force field evaluated with a definitive electronic structure method, namely all-electron (AE) coupled-cluster theory including single, double, and perturbative triple excitations [CCSD(T)] conjoined with a correlation-consistent triple- $\zeta$  atomic-orbital basis set (cc-pCVTZ). The theoretical vibrational frequencies are first-principles quantum-mechanical results devoid of empirical adjustments. For 9 of the 11 assigned bands of **1t** and **d-1t**, the mean deviation between theory and experiment is only  $8.2\text{ cm}^{-1}$ . The remaining two bands correspond to the (O–H, O–D) stretch of (**1t**, **d-1t**), for which the measured values are downshifted by  $(52, 36)\text{ cm}^{-1}$  as compared to theory. These shifts are close to those previously observed for hydroxymethylene (**9**) and may be attributed primarily to matrix effects on the H stretching frequencies.

The ultraviolet (UV) difference spectrum of **1t** (Fig. 4B) exhibits a broad weak band with maximum absorption near 393 nm (3.2 eV) that extends to around 460 nm (2.7 eV). Our computations show that this  $S_0(^1A') \rightarrow S_1(^1A'')$  band arises from a highest occupied molecular orbital ( $a'$ )  $\rightarrow$  lowest unoccupied molecular orbital ( $a''$ ) electronic transition involving excitation from the carbene lone pair into the empty p orbital of the divalent C atom. Multireference coupled-cluster computations (Mk-MRCC) with a large basis set (aug-cc-pVTZ) cement the assignment of the UV spectrum of **1t** and fully characterize the associated electronic states [supporting online material (SOM), sections 4, 5, and 8]. Upon excitation from the ground state ( $S_0$ , figs. S3 and S7) to the open-shell singlet excited electronic state ( $S_1$ ), **1t** distorts to an equilibrium structure with a widened C–C–O angle of  $124.5^\circ$  and a nonplanar C–C–O–H framework having a dihedral angle of  $112.4^\circ$ . Our best [Mk-MRCCSD(T)/aug-cc-pVTZ] computed vertical and adiabatic electronic excitation energies are  $T_v = 3.38\text{ eV}$  and  $T_0 = 2.70\text{ eV}$ , respectively, in excellent agreement with the experimental absorption spectrum.

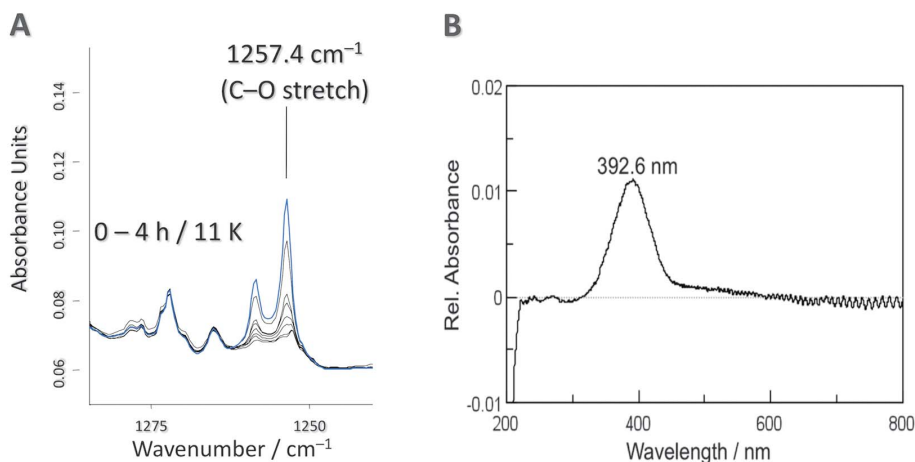
To elucidate the chemistry of methylhydroxycarbene, we computed all relevant equilibrium structures and transition states on the potential energy surface (PES) surrounding **1t** with state-of-the-art theory, specifically the AE-CCSD(T) method with a quadruple- $\zeta$  cc-pCVQZ basis set. Exhaustive quantum-mechanical focal-point analyses (FPAs) were then used to converge the salient energetic features to around  $0.1\text{ kcal mol}^{-1}$ , as demonstrated previously (**9**). Structural depictions, optimized geometries, FPA energetic tables, and complete PES schematics are provided in the SOM. The essential [1,2]H-shift energy profiles for **1** appear in Fig. 3. Our definitive computations confirm the general accuracy ( $\pm 1\text{ kcal mol}^{-1}$ ) of earlier G2 and CBS-Q computations (**18**, **24**) on the PES surrounding **1t**.

Some disparities exist among experimentally derived enthalpy changes  $\Delta H(4-3)$  between gaseous vinyl alcohol and acetaldehyde. However, calorimetric experiments on **3** (**31**) and ionization measurements on **4** (**32**) can be conjoined to obtain  $\Delta H_{298}(4-3) = 10.2 \pm 0.4\text{ kcal mol}^{-1}$ , in excellent agreement with the  $10.3\text{ kcal mol}^{-1}$  we

compute for the corresponding 298 K enthalpy difference between **3** and the cis form of **4** (**4c**). The [1,3]H-shift barrier from **4c** to **3** is prohibitively high ( $+66.3\text{ kcal mol}^{-1}$ ) for direct interconversion in our experiments. Our precisely determined energy of **1t** relative to **3** ( $+50.7\text{ kcal mol}^{-1}$ ) brings into question the value ( $+57 \pm 4\text{ kcal mol}^{-1}$ )



**Fig. 3.** PES profiles for [1,2]H-shift isomerizations of *trans*-methylhydroxycarbene (**1t**); relative energies  $\Delta H_0$  (in  $\text{kcal mol}^{-1}$ ) were computed from the convergent focal-point analyses detailed in the SOM. The bond lengths (Å) and angles ( $^\circ$ ) given for **1t** are ground-state optimum geometrical parameters given by AE-CCSD(T)/cc-pCVQZ theory (C, light gray larger atoms; O, dark gray large atom; H, light gray small atoms). The *s-cis* isomer **1c** (not shown) lies  $3.1\text{ kcal mol}^{-1}$  above **1t**. The curves are drawn quantitatively with the intrinsic reaction coordinate in mass-weighted Cartesian space as the abscissa in order to reflect the proper barrier heights and widths for the two competing reactions. Simple visual inspection thus indicates a higher H-tunneling probability for the more narrow energy profile of path **a**. The intrinsic reaction coordinate of path **b** yields *s-trans*-vinyl alcohol (**4t**) as shown; the cis form of **4** (**4c**, not shown) lies slightly lower on the energy diagram at  $-40.9\text{ kcal mol}^{-1}$ .



**Fig. 4.** (A) Part of the time-dependent IR spectrum showing the disappearance of **1t** over 4 hours at 11 K; full spectra are shown in fig. S2. (B) Experimental UV difference spectrum of **1t** at 11 K, based on absorption changes after 19 hours in the dark (see fig. S3 for raw spectra).



derived from collision-induced flowing afterglow measurements employing protonated butadiene (18). Our computations pinpoint the singlet-triplet splitting of **1t** as  $\Delta E_{\text{ST}} = -30.6 \text{ kcal mol}^{-1}$ , broadly validating the experimentally inferred value of  $\Delta E_{\text{ST}} = -28 \text{ kcal mol}^{-1}$  (18). The large  $\Delta E_{\text{ST}}$  gap emphasizes the singlet ground-state nature of **1**, as for other hydroxycarbenes (9, 14, 25). A considerable singlet-triplet separation is maintained along the entire reaction paths for **1t**  $\rightarrow$  **3** and **1t**  $\rightarrow$  **4t** (SOM, section 8), indicating that intersystem crossing is not a factor in these transformations. The key conclusion from Fig. 3 is that **1t** is blocked from **3** and **4** by large potential energy barriers of 28.0 and 22.6 kcal mol $^{-1}$ , respectively, which are much too high to be overcome by the energy available under matrix isolation conditions (11 K).

Notwithstanding the kinetic barriers surrounding **1t**, we observed rapid disappearance of this molecule after cryogenic trapping. The IR and UV bands of **1t** gradually diminished on standing at 11 K in the dark, whereas those of **3** grew. We investigated the reaction kinetics by acquiring IR spectra every 30 min while carefully shielding the matrix from all external light sources. The decay of the two most intense peaks at 3554 (OH stretch) and 1257 cm $^{-1}$  (CO stretch, Fig. 4) followed first-order kinetics with a  $t_{1/2} = 66 (\pm 5) \text{ min}$  in Ar (SOM, section 3). In stark contrast, the bands of the deuterium isotopologue **d-1t** did not change under identical conditions for extended periods of time (at least 16 hours, fig. S5). The  $t_{1/2}$  values measured for **1t** in Kr and Xe at 11 K were 196 ( $\pm 4$ ) min and 251 ( $\pm 2$ ) min, respectively. Such an increase in the H-tunneling half lives from Ar to Xe matrices has also been observed for conformational isomerizations of carboxylic acids (33).

Our kinetic experiments indicate that **1t** isolated in its ground electronic and vibrational state exhibits facile [1,2]H tunneling and that two conspicuous phenomena occur simultaneously: efficient penetration of a formidable 28.0 kcal mol $^{-1}$  barrier to yield **3**, and complete obstruction of the formation of **4** despite a much lower 22.6 kcal mol $^{-1}$  barrier. We have established the theoretical basis of these phenomena by computing pure tunneling rates for both **1t**  $\rightarrow$  **3** and **1t**  $\rightarrow$  **4t** (SOM, sections 4 and 10). The AE-CCSD(T)/cc-pCVTZ method was used to precisely map out the associated intrinsic reaction paths descending from transition states **TS**<sub>1t-3</sub> and **TS**<sub>1t-4t</sub> (Fig. 3) and to determine zero-point vibrational energies (ZPVEs) along these steepest-descent routes. Final potential energy curves for the isomerization paths were then constructed from high-quality AE-CCSD(T)/cc-pCVQZ energy points appended with the ZPVEs. Within a reaction-path Hamiltonian model, Wentzel-Kramers-Brillouin tunneling probabilities ( $\kappa$ ) were evaluated from barrier penetration integrals ( $\theta$ ) computed numerically from our highly accurate electronic structure results. This first-principles approach to quantifying H-tunneling rates has proved very effective for other hydroxycarbenes (9, 14).

The reaction modes of **1t** that lead to **TS**<sub>1t-3</sub> and **TS**<sub>1t-4t</sub> have harmonic vibrational frequencies ( $\omega_0$ ) of 1346 and 736 cm $^{-1}$ , respectively; and 0 K energies ( $\epsilon$ ) for barrier collisions may be ascribed as  $\omega_0/2$  in each case. Tunneling rates were computed as the product of the transmission coefficient [ $\kappa(\epsilon)$ ] and the classical rate ( $\omega_0$ ) at which the reactant hits the barrier. This theoretical analysis yields a tunneling  $t_{1/2}$  of 71 min for **1t**  $\rightarrow$  **3**, in close agreement with the observed rate of decay. Moreover, the computed  $t_{1/2}$  for **1t**  $\rightarrow$  **4t** is 190 days, nicely explaining why vinyl alcohol is not the preferred product of methylhydroxycarbene isomerization, despite the lower barrier for formation of **4t**. Our computational procedure also yields a tunneling  $t_{1/2}$  for **d-1t** of 4000 years, consistent with the observed shutdown of the methylhydroxycarbene decay mechanism upon deuterium substitution.

The penetration integral  $\theta(\epsilon)$  and hence the tunneling probability  $\kappa(\epsilon) \approx e^{-2\theta(\epsilon)}$  depend principally on three factors (7, 34): the width of the barrier  $w(\epsilon)$ , the square root of the difference between the overall barrier height ( $V^*$ ) and  $\epsilon$ , and the square root of the effective mass. Analytic mathematical expressions relating  $\theta$  to these three factors are given in the SOM for several common barrier models. As shown in Fig. 3, the reduced width of the **1t**  $\rightarrow$  **3** barrier amply overcomes the reduced height of the **1t**  $\rightarrow$  **4t** barrier, resulting in respective  $\kappa$  values of  $4 \times 10^{-18}$  and  $2 \times 10^{-21}$ . In brief, barrier width trumps barrier height (34) because  $\theta$  scales linearly with the former but only as the square root of the latter.

The relative widths of the barriers for **1t**  $\rightarrow$  **3** and **1t**  $\rightarrow$  **4t** can be traced directly to geometric requirements for these chemical transformations (fig. S6). Variations in the potential energy curves for both reactions persist over the entire range of arc lengths in Fig. 3, and the total arc length for the multidimensional **1t**  $\rightarrow$  **4t** reaction path is 19% greater than in the **1t**  $\rightarrow$  **3** case. Accordingly, the respective  $\theta$  values exhibit almost the same proportion. The increased arc length for **1t**  $\rightarrow$  **4t** has two main causes. First, the migrating H must travel 2.42 Å to its final position in the **1t**  $\rightarrow$  **4t** reaction but only 2.11 Å in the **1t**  $\rightarrow$  **3** case. Second, a concomitant conformational change is required for **1t**  $\rightarrow$  **4t** in order to bring all atoms into the same plane in the product; in particular, a second methyl H in **1t** must shift from 53° out of the C–C–O plane to a coplanar position in **4t**.

Methylhydroxycarbene demonstrates the need to consider tunneling control in addition to classical kinetic or thermodynamic control in order to analyze, predict, and understand the full diversity of chemical reactivity. Tunneling control may be considered a type of nonclassical kinetic control in which the decisive factor is not the lowest activation barrier. Chemical reactions governed by tunneling mechanisms can be a general phenomenon, especially if H transfer is involved, and such processes need not be restricted to cryogenic temperatures. Much more research is necessary to reveal the possibilities of tunneling control in

fields ranging from biochemistry to materials science.

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## Supporting Online Material

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# Origin and Evolution of Prebiotic Organic Matter As Inferred from the Tagish Lake Meteorite

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The complex suite of organic materials in carbonaceous chondrite meteorites probably originally formed in the interstellar medium and/or the solar protoplanetary disk, but was subsequently modified in the meteorites' asteroidal parent bodies. The mechanisms of formation and modification are still very poorly understood. We carried out a systematic study of variations in the mineralogy, petrology, and soluble and insoluble organic matter in distinct fragments of the Tagish Lake meteorite. The variations correlate with indicators of parent body aqueous alteration. At least some molecules of prebiotic importance formed during the alteration.

Carbonaceous chondrite meteorites are samples of kilometer-sized primitive asteroids that preserve to varying degrees the initial solid components of the solar protoplanetary disk [or nebula (*1*)]. As such, these meteorites are samples of the material that took part in planet formation nearly 4.6 billion years ago. The chondrites also preserve a record of the processes that occurred in their asteroidal parent bodies, such as thermal metamorphism, aqueous alteration, and impact brecciation (*1*). Organic matter composes up to several weight percent of carbonaceous chondrites and includes macromolecular material and a variety of simpler molecules (*2*) that are generally referred to as insoluble organic matter (IOM) and soluble organic matter (SOM), respectively, because of their relative solubilities in typical solvents (*3, 4*). Organic matter in carbonaceous chondrites shares characteristics with material from other primitive extraterrestrial samples, including interplanetary dust particles (IDPs), samples of comet 81P/Wild 2 (*5, 6*), and some Antarctic micrometeorites (*7*). The common features of IOM from carbonaceous chondrites and comets suggest that there was a common source of such organic matter—the outer solar nebula and/or the interstellar medium—and that the

diversity of organic matter in meteorites is the result of variable degrees of parent body modification (*8*).

Earth's carbon was provided by the accretion of early solar system solids. The accretion of meteorites and other asteroidal and cometary material by the early Earth may have been a source of intact organic matter that was necessary for the advent of life (*9*). Carbonaceous chondrite SOM includes molecules of prebiotic interest such as amino acids, nucleobases, monocarboxylic acids (MCAs), sugars, and polycyclic aromatic hydrocarbons (*3*). Some of these compounds may be the result of hydrothermal alteration of IOM in the meteorite parent bodies (*10–12*), but which compounds formed in this manner is an open question.

Here we report on IOM and SOM in several individual stones of the Tagish Lake meteorite shower (*13*) that have experienced different levels of hydrothermal alteration (*14*). The meteorite is an ungrouped type 2 carbonaceous chondrite (it has affinities to both CI and CM meteorites) consisting of chondrules set in a fine-grained matrix that is dominated by serpentine and saponite clay minerals (*15*), and it has been linked to the primitive D-type asteroids (*16*). Lithological variability on the scale of individual stones may be attributable to different conditions of alteration and/or impact brecciation (*15*). The Tagish Lake meteorite contains a high concentration of or-

ganic matter, nearly 3 weight percent (wt %) (*17*). An unusual distribution of soluble organic compounds that are dominated by carboxylic and sulfonic acids, with only trace (part-per-billion) levels of amino acids, has previously been reported for the Tagish Lake meteorite, suggesting a distinct pathway of organic synthesis as compared to CI and CM meteorites (*18, 19*). Sub-micrometer-scale carbonaceous globules that are often substantially enriched in <sup>15</sup>N and D and are thought to have formed in the interstellar medium or the cold outer solar nebula were previously identified in the Tagish Lake meteorite (*5, 20*), demonstrating the preservation of such material in spite of parent body alteration.

Terrestrial contamination and modification, both abiotic and biotic, are perennial concerns in the study of meteorite organics. The first Tagish Lake meteorite specimens fell on a frozen lake, were collected without hand contact within a few days of the fall, and have been kept frozen ever since (*21*), providing an opportunity for the study of organic matter in a pristine meteorite sample. Much of what is known about the Tagish Lake meteorite derives from studies of this pristine material (*18, 22*). However, only a handful of the 48 pristine stones have been examined in detail (*21*). We selected four specimens from among these stones on the basis of their macroscopic properties, in order to carry out a systematic study of the variations in organic matter in this meteorite and to test whether variations in IOM or SOM correlate with petrologic differences. We processed subsamples of each of the four specimens (5b, mass 4.3 g; 11h, 6.2 g; 11i, 4.7 g; and 11v, 5.6 g) in parallel, providing extracts for the analysis of SOM and IOM separates, material for x-ray diffraction, and polished mounts for microbeam analyses (*13*).

All four specimens are composed of olivine- and pyroxene-bearing chondrules and chondrule-like objects, compact lithic fragments, and isolated olivine or pyroxene grains, set in a fine-grained porous matrix dominated by clays, sulphides, magnetite, and carbonates. Based on the relative proportions of porous matrix and framboidal magnetite (*15*), and the increasing replacement of chondrule glass by phyllosilicates (*23*), the degree to which the specimens have undergone aqueous alteration is in the order 5b < 11h << 11i. Specimen 11v, which consists of disaggregated material collected from the lake ice surface, is heterogeneous on the microscale, comprising clasts whose petrologic

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**Table 1.** Summary of results of IOM analysis of Tagish Lake specimens. See (*41*). Previous data are from (*8*).

| Sample                | Previous | 11v      | 11i      | 11h   | 5b       |
|-----------------------|----------|----------|----------|-------|----------|
| C (wt %)              | ~2       | 1.77(9)  | 1.82(4)  | 1.86  | 1.6(3)   |
| H/C (at.)             | 0.337    | 0.44(1)  | 0.51(2)  | 0.594 | 0.72(4)  |
| N/C (at.)             | 0.043(2) | 0.041(1) | 0.042(2) | 0.042 | 0.042(2) |
| δ <sup>13</sup> C (‰) | −14.2(1) | −13.3    | −13.3(1) | −14.3 | −14.7(2) |
| δ <sup>15</sup> N (‰) | 73(2)    | 58(2)    | 53(1)    | 57    | 57(4)    |
| δD (‰)                | 596(4)   | 815(25)  | 992(15)  | 1470  | 1844(10) |



characteristics cover the range seen in the other three specimens. The macroscopic differences among the specimens are attributable to the proportions of the various components, as well as matrix grain size. For example, 11i, which is very dark and tends to shed a residue of black dust, has a lower proportion of chondrules and a smaller average matrix grain size ( $<5\ \mu\text{m}$ ).

Isotopic and chemical analyses of bulk IOM separates from each of the four specimens (Table 1 and Fig. 1A) show that the largest variations are in the H/C ratios and H isotopic compositions ( $\delta\text{D}$ ); variations in N isotopic compositions and in C in IOM as a proportion of the rock are negligible. C isotopic compositions show a small but substantial increase in the order  $5b > 11h > 11i \sim 11v$  (Table 1). The variations in H/C and  $\delta\text{D}$  observed in IOM in these specimens span almost the entire range found among the different carbonaceous chondrite groups (Fig. 1A). This lends credence to the suggestion that the variation in IOM elemental and isotopic compositions found in chondrites is the result of parent body modification of a common precursor (8). Furthermore, there is a linear correlation between H/C ratios and  $\delta\text{D}$  values (Fig. 1). Solid-state  $^{13}\text{C}$  and  $^1\text{H}$  nuclear magnetic resonance spectroscopy and carbon x-ray absorption near-edge spectroscopy [C-XANES (24)] (13) indicate that the decrease in the H/C ratio is accompanied by an increase in the proportion of aromatic C in the IOM as well as a considerable increase in aromatic substitution, probably aromatic condensation (13). The change in H/C was not accompanied by a substantial loss of C (Table 1), which may indicate that the aliphatic component in the Tagish Lake meteorite was converted into aromatic C, while undergoing H isotopic exchange with the altering fluid and/or preferential D loss. This apparently facile transformation is unexpected. It is most likely caused by hydrothermal alteration, as is observed in experiments involving hydrous pyrolysis or reaction with water at elevated temperature and pressure (11, 25), and differs from the scenario in which aliphatic C is selectively removed through reaction with an oxidant (26).

High-spatial-resolution secondary ion mass spectroscopic (SIMS) measurements reveal that the isotopic differences observed in bulk IOM residues extend to submicrometer scales. IOM from sample 5b shows not only a higher average D/H ratio but also a much higher proportion of very D-rich submicrometer-sized isotopic hot spots (Fig. 1B) with more extreme D/H ratios than those from 11v [maximum  $\delta\text{D} \sim 20,000$  per mil (‰) in 5b versus  $\sim 7000$ ‰ in 11v]. These observations suggest that parent body alteration has substantially removed D, decreasing the D/H ratio on all spatial scales and reducing the number of hot spots. Similar variations in D enrichments and abundances between chondrites have been observed before, but never in a single chondrite. In contrast, the N isotopic distributions are similar except that 5b contains about twice the number density of  $^{15}\text{N}$  hot spots (with  $\delta^{15}\text{N}$  in both residues up to  $\sim 800$ ‰). This difference in behavior of H and N isotopes supports observations in previous studies that D and  $^{15}\text{N}$  enrichments in IOM tend to be decoupled (5). Isotopic hot spots are, in many cases, associated with carbonaceous nanoglobules (5, 20). Transmission electron microscope (TEM) examinations indicate that IOM from sample 5b has a significantly higher fraction (7.5%) of nanoglobules than does IOM from 11v (0.9%) (13). C-XANES (24) indicates the presence of two chemical classes of nanoglobules, one with a C functional group distribution similar to that in nonglobular IOM and one dominated by aromatic functionality (13). Aromatic-type nanoglobule spectra are seen in a higher fraction of nanoglobules from 11v as compared to 5b [50% versus 20% (13)]. Taken together, the SIMS, TEM, and XANES results suggest that  $^{15}\text{N}$ -rich nanoglobules have been preferentially destroyed in specimen 11v by hydrothermal alteration. Moreover, the higher fraction of highly aromatic nanoglobules in the more altered sample supports the conclusion from the bulk data that the alteration largely affects the aliphatic component of the IOM.

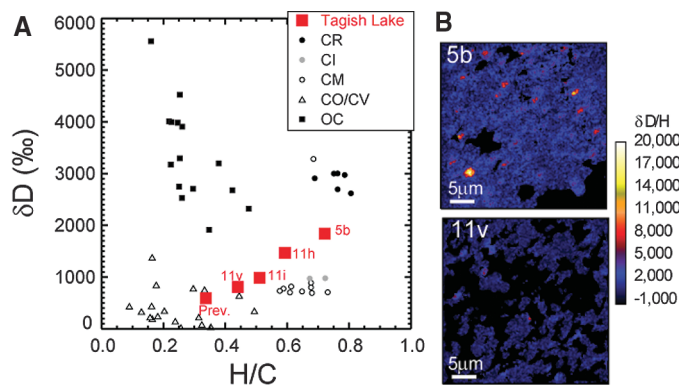
Based on IOM results, the degree of alteration reflected by the Tagish Lake specimens is  $5b <$

$11h < 11i < 11v$ , which is consistent with the order inferred petrologically. Within this context, we examined the results of the SOM analysis to determine whether the hydrothermal alteration has resulted in the formation, modification, or destruction of soluble organic molecules and to elucidate the relationship between IOM and SOM during the alteration.

MCAs dominate the water extracts of the Tagish Lake meteorite. MCAs, such as formic and acetic acids, play essential roles in biochemistry (11, 27, 28); higher homologs are the fatty acids that self-assemble into membrane-bound vesicles in meteorite extracts and are the possible precursors of cell membranes (29). We identified 11 MCAs in all specimens, including most of the members of the homologous series of linear, saturated MCAs from  $\text{C}_1$  to  $\text{C}_{10}$ . One or two branched isomers were detected in all specimens with the exception of 5b, in which 17 branched isomers were detected, in addition to the 11 linear MCAs. Numerous alkyl-substituted phenols were also found exclusively in 5b. Although, as in previous studies,  $\delta^{13}\text{C}$  values are generally consistent with terrestrial values, these MCA hydrogen isotopic compositions are D-enriched, consistent with an extraterrestrial origin (2): As measured in 5b,  $\delta\text{D}$  (acetic), 247‰;  $\delta\text{D}$  (formic/propanoic), 708‰;  $\delta\text{D}$  (butanoic), 562‰;  $\delta\text{D}$  (isopentanoic), 697‰ (13). The observed concentrations of these low-molecular-weight MCAs are unusually high relative to those seen in other studies of carbonaceous chondrites [including Tagish Lake (18)], ranging from 42 to 250 parts per million (ppm) for formic and acetic acid (13). We attribute these large concentrations to the preservation of the meteorite below  $0^\circ\text{C}$  since its recovery, which has minimized the loss of volatile organics, such as formic acid, as well as the specifics of the analytical methods (13). In nearly all specimens, the concentrations of the straight-chain MCAs decrease in a logarithmic manner as the C number increases, with the exception of 5b, in which the acetic acid concentration exceeds that of formic acid. The  $\delta^{13}\text{C}$  values of MCAs differ among the specimens (Fig. 2). All specimens have common  $\delta^{13}\text{C} \sim -20$ ‰ for formic acid, and higher homologs approach a constant value of  $\sim -5$ ‰ (average nonanoic acid =  $-26 \pm 2$ ‰) with increasing C number. The largest differences are observed in acetic acid, which ranges from +8‰ (11h) to  $-36$ ‰ (5b). Of particular note is specimen 11h, which shows a decrease in  $\delta^{13}\text{C}$  with increasing C number (Fig. 2).

The differences in MCAs among the Tagish Lake specimens may be explained by differing degrees of parent body modification. With the exception of formic acid, specimens 5b and 11h contain the highest concentrations of MCAs, 2 to 10 times greater than concentrations in 11i and 11v (13), attributable to loss or destruction of these water-soluble compounds during progressive parent body alteration. The high proportion of branched isomers in specimen 5b suggests that it preserves a more primary suite of compounds

**Fig. 1. (A)** Plot of H/C, a measure of the degree of aliphatic character, against H isotopic composition for the Tagish Lake specimens, including data on the Tagish Lake meteorite from previous work (8). Also shown are representative data from other chondrite groups after (8), including ordinary chondrites (OC). For reference, the H/C value of an aliphatic molecule with infinite chain length



is 2; aromatic organic matter has a maximum H/C = 1 (benzene), and approaches low values ( $\sim 0.1$ ) as the number of fused aromatic rings approaches infinity. (B) Maps of  $\delta\text{D}/\text{H}$  values of IOM separates from Tagish Lake specimens 5b and 11v, derived from H and D raster ion images acquired with a Cameca NanoSIMS 50L ion microprobe.

(2). The MCA pattern for 11h shows a trend of decreasing  $\delta^{13}\text{C}$  with increasing C number, comparable to results for Murchison (30). Whereas this trend has been attributed to the preservation of the signature of kinetically controlled C addition in MCA synthesis, which takes place in cold, interstellar, or nebular environments (31), our results, which suggest that specimen 11h is more altered than 5b, imply that such a pattern may be a secondary signature. One possible explanation for the pattern in this case is the preferential exchange of MCA carboxyl C with inorganic C during hydrothermal processing, analogous to the process that occurs in oil-prone source rocks on Earth (32). In the Tagish Lake meteorite, the presence of carbonate  $\delta^{13}\text{C} \sim 67\text{‰}$  (17) may provide a source of isotopically enriched carbonate for such exchange. Notably, formic acid concentration and C isotopic composition remain relatively constant among the specimens (13), which suggests that they are relatively unaffected by aqueous alteration (10) and may be inherited from preaccretionary material.

Amino acid concentrations and enantiomeric excesses in the Tagish Lake specimens provide further evidence of the influence of parent body aqueous alteration on SOM. We determined the distribution and enantiomeric abundances of the one- to six-C aliphatic amino acids found in extracts of specimens, 5b, 11h, and 11i by ultra-performance liquid chromatography fluorescence detection and time-of-flight mass spectrometry (33). We measured stable C isotope analyses of the most abundant amino acids in 11h with gas chromatography coupled with quadrupole mass spectrometry and isotope ratio mass spectrometry. The total abundances of amino acids decrease in the order 11h (5.6 ppm) > 5b (0.9 ppm) > 11i (0.04 ppm). The abundances of many amino acids in 11i were below the analytical detection limit (<1 part per billion), which is consistent with a much higher degree of alteration experienced by 11i as compared to 11h and 5b. The abundance of the nonprotein amino acid  $\alpha$ -aminoisobutyric acid in specimen 11h was 0.2 ppm, approximately 200 times higher than previously measured in two different Tagish Lake meteorite samples (18, 19). Glycine is the most abundant amino acid in 11h and has a C isotope value of  $\delta^{13}\text{C} = +19\text{‰}$ , which falls well outside the range for terrestrial organic C of  $-6$  to  $-40\text{‰}$  (34) and is consistent with an extraterrestrial origin.

The enantiomeric ratios of alanine,  $\beta$ -amino-*n*-butyric acid, and isovaline in 11h were racemic within uncertainties ( $D/L = 1$ ), providing additional evidence of an extraterrestrial origin for these amino acids. In contrast to specimen 11h, nonracemic isovaline was detected in 5b, with an L-enantiomeric excess of  $\sim 7\%$ , and no isovaline was identified in 11i above the detection limit. Although the mechanism of enrichment remains unclear, it has been previously shown that L-isovaline enantiomeric excesses (ee's) and the ratio of  $\beta$ -alanine to glycine both increase relative to the degree of aqueous alteration for many

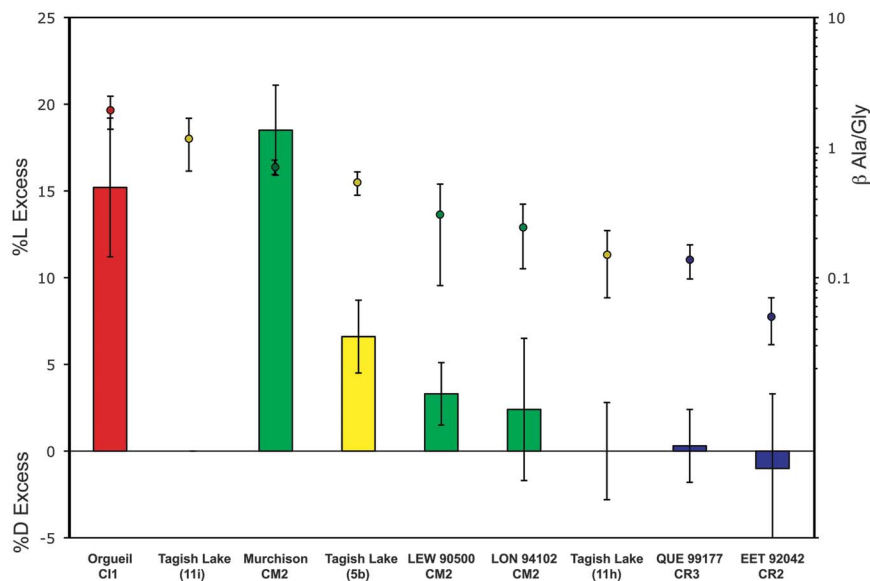
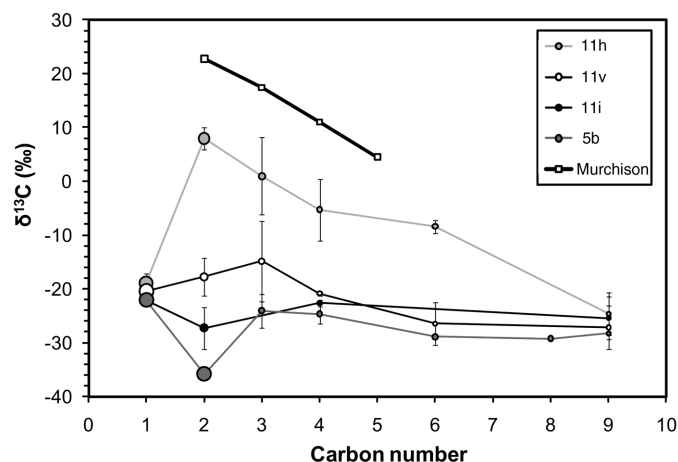
carbonaceous chondrite groups (33, 35). Although the data for specimen 11i relative to 11h or 5b fit this trend (Fig. 3), in detail the sequence of alteration for 5b and 11h based on these criteria suggests that 5b is more altered than 11h, in contrast to the result from petrography and IOM. This result suggests that other factors may influence ee's and the  $\beta$ -alanine/glycine ratio that are apparent in the Tagish Lake meteorite. The higher ratio of  $\beta$ -alanine to glycine in 5b ( $\sim 0.6$ ) as compared to 11h ( $\sim 0.2$ ) may be due to enhanced production of glycine during aqueous alteration of 11h via reactions involving hydroxy acids known to be present in SOM (36, 37). A study of L-isovaline ee's in Murchison specimens showed a range of ee values from 0 to 15%, roughly correlative with the abundance of hydrated

minerals in the samples, indicating the role of multiple, complex, parent body synthetic processes in amino acid formation (38). The amino acids in Tagish Lake 11h, including ee's and overall abundance, may therefore be interpreted as reflecting a secondary pulse of amino acid formation resulting from hydrothermal alteration on the Tagish Lake parent body, which overprinted any original ee's with a racemic mixture.

Substantial heterogeneity is preserved within the Tagish Lake meteorite, especially in terms of organic matter. The correlation between differences in organic matter properties and indicators of hydrothermal alteration indicates that the processes were active after accretion onto the parent body. In this scenario, chondritic components, including D- and  $^{15}\text{N}$ -rich IOM that is best pre-

**Fig. 2.** C isotopic composition of monocarboxylic acids in the Tagish Lake meteorite. Uncertainties represent the standard deviation of three injections for each sample. For measurements with low amplitude (such as those of nonanoic or decanoic acid) we used a value of 4‰, which is based on the accuracy achieved for standards run with low concentrations. Also shown are the results from (31) for Murchison monocarboxylic acids.

Symbol size reflects relative concentration (13).



**Fig. 3.** L-isovaline ee's (bars) and  $\beta$ -alanine/glycine ratios (circles) in Tagish Lake meteorite specimens 11h, 5b, and 11i (shown in yellow), compared with results from CI (red), CM (green), and CR (blue) chondrites of differing degrees of aqueous alteration [data from (33)]. The percentage of L excess is defined as  $L_{ee} = L\% - D\%$ , with a negative value corresponding to a D excess. LEW, Lewis Cliff; LON, Lonewolf Nunataks; QUE, Queen Alexandra Range; EET, Elephant Moraine.



served in 5b, were accreted, along with (presumably) amino acid precursors. The  $\alpha$ -amino acids were probably produced during alteration on the Tagish Lake parent body, presumably by Strecker synthesis (37, 39), although other formation mechanisms for both  $\alpha$  and other amino acids before their incorporation in the parent body have been suggested (40). Modest alteration may have produced light acetic acid and an initial complement of MCAs from IOM, by analogy with experiments (11), as well as a slight ee in isovaline, to provide the SOM characteristics observed in 5b. These components were then modified on the parent body through further hydrothermal alteration, resulting in reduction of aliphatic character and D/H in IOM, exchange of isotopically heavy C with MCA carboxyl C, production of glycine, and a fresh influx of racemic amino acids, as represented by organic matter in 11h. By analogy with MCAs, the exchange of isotopically heavy C with amino acid carboxyl C may explain the positive  $\delta^{13}\text{C}$  values of amino acids in 11h (such as glycine). The increase in IOM  $\delta^{13}\text{C}$  with the degree of alteration (Table 1) is consistent with the loss of isotopically lighter C, associated with aliphatics, such as MCAs in 11i and 11v. Further hydrothermal alteration resulted in further modification of IOM and decreases in overall concentration of MCAs in 11i and 11v and a nearly complete loss of amino acids in 11i. The conditions of hydrothermal alteration inferred by analogy with experiments, especially temperature ( $\sim 300^\circ\text{C}$ ) (10, 11, 25), are at odds with the mineralogy and preservation of volatile organic compounds, which provide an upper limit of  $\sim 150^\circ\text{C}$  (23). The Tagish Lake specimens may therefore have experienced alteration at lower temperatures than those in the experiments, with the more extensively altered samples having been subjected to longer periods of alteration, higher temperatures, and/or higher water/rock ratios (11).

13. Information on materials and methods is available as supporting material on Science Online.
14. Hydrothermal alteration occurred early in the history of the carbonaceous chondrite parent bodies owing to accumulation of the heat of radioactive decay, so that liquid water was transiently present and percolated through the mineral matrix. The evidence for this process is preserved in mineral alterations. Furthermore, in the interior of the parent body, the temperature and pressure can rise high enough to produce hydrolysis of organic material.
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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1304/DC1  
Materials and Methods  
Figs. S1 to S5  
Tables S1 to S4  
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## Activation of Visual Pigments by Light and Heat

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Vision begins with photoisomerization of visual pigments. Thermal energy can complement photon energy to drive photoisomerization, but it also triggers spontaneous pigment activation as noise that interferes with light detection. For half a century, the mechanism underlying this dark noise has remained controversial. We report here a quantitative relation between a pigment's photoactivation energy and its peak-absorption wavelength,  $\lambda_{\text{max}}$ . Using this relation and assuming that pigment activations by light and heat go through the same ground-state isomerization energy barrier, we can predict the relative noise of diverse pigments with multi-vibrational-mode thermal statistics. The agreement between predictions and our measurements strongly suggests that pigment noise arises from canonical isomerization. The predicted high noise for pigments with  $\lambda_{\text{max}}$  in the infrared presumably explains why they apparently do not exist in nature.

Our visual system has an extremely high sensitivity to light under dark-adapted conditions (1). This feat requires a photo-

transduction mechanism with high amplification (2) and a thermally quiet visual pigment for minimizing noise. Thermal energy is a double-edged

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sword: It extends the spectral sensitivity of a pigment to long wavelengths by overcoming the energy deficit of a long-wavelength photon to drive pigment excitation (3–6), but it also triggers pigment activation occasionally in darkness to produce noise (7). More than 50 years ago, Barlow (8) proposed that pigments with longer peak-absorption wavelengths ( $\lambda_{\max}$ ) are noisier and thus less suitable for dim-light detection. Although qualitatively validated (9–14), Barlow's seminal hypothesis lacks a mechanistic underpinning. First, no prior relation exists between  $\lambda_{\max}$  and thermal activation, which impedes any quantitative prediction of pigment noise. Second, controversy continues about whether the pigment noise originates from an isomerization reaction and, if so, whether it is canonical isomerization (i.e., governed by the same ground-state isomerization energy barrier as in photoisomerization) (15–17). Finally, despite the rhodopsin noise measured long ago (7), noise measurements for cone pigments have begun to emerge only recently (10, 11, 13, 14), thus making any comprehensive pigment-noise theory untestable until now. We report here final success in understanding this fundamental problem in vision.

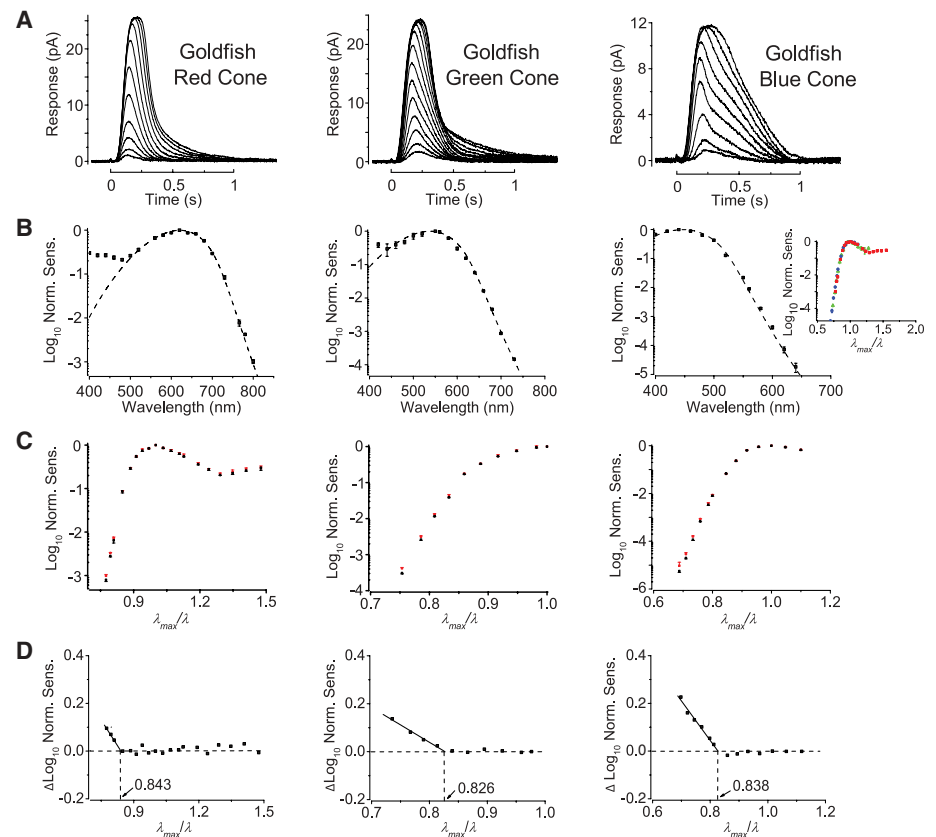
The involvement of thermal energy in pigment excitation by long-wavelength photons is implicit in the characteristic descent of a pigment's spectrum at such wavelengths (3–5) and is directly revealed by an increase in relative absorption of these photons at a higher temperature (6). From the critical wavelength ( $\lambda_c$ ) above which a temperature effect begins to appear, the photoactivation energy,  $E_a^P$ , can be obtained (fig. S1) (18). In this way, Ala-Laurila *et al.* (19, 20) have used microspectrophotometry and electroretinography to show that an  $E_a^P$  change is well correlated with a  $\lambda_{\max}$  shift produced by a chromophore switch from 11-*cis*-retinal ( $A_1$ ) to 11-*cis*-3-dehydroretinal ( $A_2$ ) in a pigment (18), but that this correlation is otherwise weak across pigments with different opsins. We reexamined this question, with the more precise suction-pipette recording (18), on diverse rod and cone pigments spanning phyla and  $A_1$  and  $A_2$  chromophores.

Figure 1A shows flash responses from dissociated goldfish red-, green- and blue-sensitive cones. Fitting their action spectra (Fig. 1B) with the  $A_2$ -pigment spectral template (21) gave  $\lambda_{\max}$  values of 620, 537, and 447 nm, respectively. When logarithmic normalized sensitivity (i.e.,

response per incident photon) was plotted against reciprocal normalized wavelength ( $\lambda_{\max}/\lambda$ ), the three spectral-sensitivity curves superposed well and showed a linear descent toward long wavelengths (Fig. 1B, inset), consistent with previous work (3–5, 22). Raising the temperature increased the relative sensitivity at long wavelengths, but not near  $\lambda_{\max}$  or at shorter wavelengths (Fig. 1C) (18). From the difference in sensitivity between the two temperatures plotted against  $\lambda_{\max}/\lambda$  (Fig. 1D) (19, 23), we obtained, by interpolation, a  $\lambda_{\max}/\lambda_c$  ratio of 0.843, 0.826, and 0.838, respectively, for the three cone types. The same experiment on *Bufo* and larval salamander red rods, as well as mouse rods, gave a  $\lambda_{\max}/\lambda_c$  ratio of 0.843, 0.830, and 0.840, respectively (Fig. 2, A and B) (18). We also measured mouse S [ultraviolet (UV)-sensitive] cone pigment, which has an unusual, unprotonated Schiff base (24) and thus

potentially different photoisomerization energetics. Because a UV pigment is typically coexpressed in native cones with a longer- $\lambda_{\max}$  pigment (25, 26), we used an engineered mouse line in which the rods express only the UV cone pigment (18, 27). Again, we found a  $\lambda_{\max}/\lambda_c$  value of 0.841 (Fig. 2C).

From the above data, the mean  $\lambda_{\max}/\lambda_c$  value is  $0.837 (\pm 0.007, \text{SD})$ . Separately, a  $\lambda_{\max}/\lambda_c$  of  $0.842 (\pm 0.009, \text{SD})$  (table S1) was obtained by directly estimating  $E_a^P$  (hence  $\lambda_c$ ) from the same data (18). The overall mean  $\lambda_{\max}/\lambda_c$  from both methods is 0.84. Thus,  $E_a^P = hc/\lambda_c \approx 0.84hc/\lambda_{\max}$  ( $h$ , Planck's constant;  $c$ , speed of light), regardless of whether it is an  $A_1$  or  $A_2$ , a UV- or non-UV-sensitive, or a rod or cone pigment. This general relation allows us to deduce  $E_a^P$  for any pigment of known  $\lambda_{\max}$ . The constancy of  $\lambda_{\max}/\lambda_c$  no doubt contributes to the stereotypic



**Fig. 1.** Effect of temperature on spectral sensitivity of goldfish cone pigments. **(A)** Flash responses from a single cell (averaged responses, 10-msec flash at time zero, 23°C). **(B)** Logarithmic normalized sensitivity [obtained from dim-flash responses as illustrated in (A)] plotted against wavelength. Average  $\pm$  SEM (8, 6, and 5 cells, respectively); the SEMs were too small to be discernible at most wavelengths. The dotted curves are fits with the  $A_2$  pigment spectral template (21) with  $\lambda_{\max}$  of 620, 537, and 447 nm, respectively. The inset at right is an overlay of the three action spectra (with colors corresponding to respective cone types), plotted in this case against the reciprocal of normalized wavelength ( $\lambda_{\max}/\lambda$ ), to show the common shape (5, 22). **(C)** Logarithmic normalized sensitivity plotted against  $\lambda_{\max}/\lambda$  at two temperatures. Average  $\pm$  SEM. Cold temperature (black) was 14°C for red- (7 cells), 13°C for green- (11 cells) and 13°C for blue-sensitive cones (12 cells). Warm temperature (red) was 28°C for all three cone types (8, 7, and 8 cells, respectively). **(D)** Difference in logarithmic normalized sensitivity against  $\lambda_{\max}/\lambda$  between the two temperatures calculated from (C). Horizontal dashed line represents essentially no temperature dependence. Linear regression from the temperature dependence at long wavelengths (solid line) intersects the horizontal line at 0.843, 0.826, and 0.838, respectively.

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descent of the action spectra at long wavelengths when plotted against  $\lambda_{\max}/\lambda$  (Fig. 1B, inset).

To predict the thermal noise of visual pigments, we adhered to the parsimonious notion that thermal activation reflects canonical isomerization of the pigment—that is, it is dictated by the same ground-state energy barrier for isomerization,  $E_a^T$ , as in photoisomerization (fig. S2). We adopted the following statistical-mechanical distribution (6, 28–30)

$$f_{\geq E_a^T} = e^{-\frac{E_a^T}{RT}} \sum_m \frac{1}{(m-1)!} \left( \frac{E_a^T}{RT} \right)^{m-1} \quad (1)$$

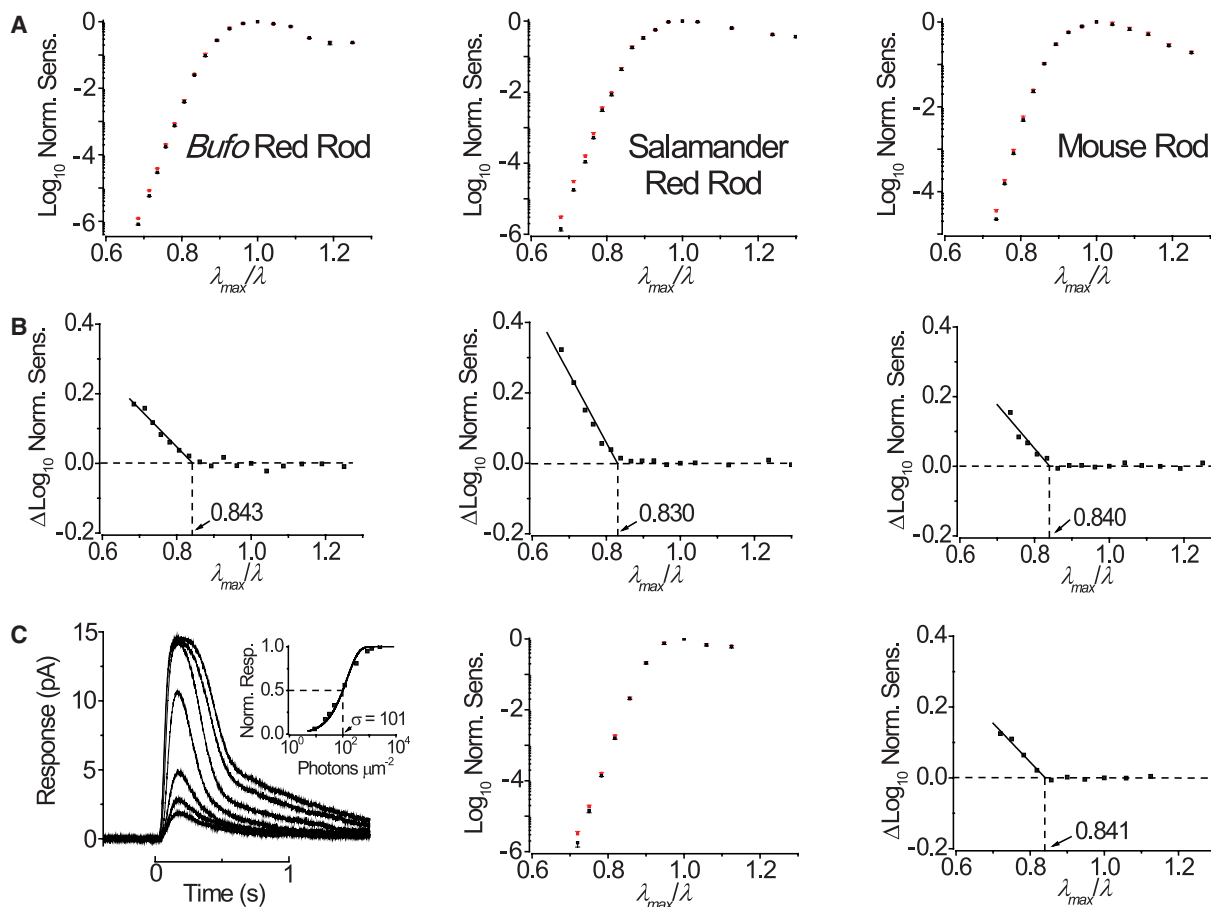
which describes a pigment molecule's probability,  $f_{\geq E_a^T}$ , of having relevant thermal energy  $\geq E_a^T$ , and thus being able to isomerize thermally. Here,  $R$  is the universal gas constant,  $T$  is absolute temperature, and  $m$  is the number of molecular vibrational modes contributing thermal energy to pigment activation (18). The Boltzmann

distribution,  $f_{\geq E_a^T} = e^{-\frac{E_a^T}{RT}}$ , corresponds to the special case of  $m = 1$  in Eq. 1, and, strictly speaking, applies only to an ideal gas. Equation 1 is thus a more general distribution, allowing the possibility of thermal energy coming from multiple vibrational modes in a complex molecule but without requiring knowledge of the molecular details of the modes or the nature of their energy transfer.  $E_a^T$  is an unknown but should be  $>35$  kcal mol<sup>-1</sup> for rhodopsin [ $\sim 40$  kcal mol<sup>-1</sup> or more (31–34)], because an early photoisomerized state, bathorhodopsin, is already at  $\sim 35$  kcal mol<sup>-1</sup> above dark rhodopsin (fig. S2) (31). We let  $E_a^T = \alpha E_a^P$ , where  $\alpha$  is a proportionality constant  $\leq 1$ ; the initial possibility of  $\alpha > 1$  (31) has been disfavored (32–34).

We began calculations with  $\alpha = 1$ ; thus,  $E_a^T = E_a^P = 0.84hc/\lambda_{\max}$ . For rhodopsin with  $\lambda_{\max} = 500$  nm, we obtain  $E_a^T = 48.03$  kcal mol<sup>-1</sup>. Previously, an apparent thermal activation energy [ $E_a^{T(\text{app})}$ ] of 21.9 kcal mol<sup>-1</sup> was found for rho-

dopsin (7), based on the Arrhenius equation, in which the Boltzmann distribution is implicit. This discrepancy between the  $E_a^T$  and  $E_a^{T(\text{app})}$  values has prompted the suggestion (15–17) that thermal activation somehow bypasses the energy barrier  $E_a^T$  associated with photoisomerization. This ad hoc assumption becomes unnecessary with  $m > 1$  in Eq. 1. The  $m$  value can be obtained from the relation  $E_a^T - E_a^{T(\text{app})} = (m-1)RT$  (6, 28, 29), giving  $m = 45$  at 23°C (18). This  $m$  value is nominal, based on each vibrational mode of the molecule contributing a nominal energy of  $kT$  (where Boltzmann's constant  $k = R/N_A$ , with  $N_A$  as Avogadro's number).

With Eq. 1, we predicted the relative thermal noise rates for diverse pigments at 23°C (our reference temperature) by using the respective  $E_a^T$  values calculated from their  $\lambda_{\max}$  values, as described above, and keeping  $m = 45$  across pigments because their chromophore is essentially the same, whether A<sub>1</sub> or A<sub>2</sub>. The absolute rate is



**Fig. 2.** Effect of temperature on spectral sensitivity of rhodopsins and S (UV-sensitive) cone pigment. (A) Temperature effects on the action spectra of the three rhodopsins, displayed in same format as in Fig. 1C. Cold temperature (black) was 14°C for *Bufo* (9 cells), 13°C for salamander (11 cells), and 25°C for mouse (8 cells). Warm temperature (red) was 25°C (10 cells), 25°C (11 cells), and 37.5°C (9 cells), respectively. (B) Difference spectrum between the two temperatures obtained from (A), giving  $\lambda_{\max}/\lambda_c$  of 0.843, 0.830, and 0.840, respectively. (C) Temperature effect on the action spectrum of mouse S cone

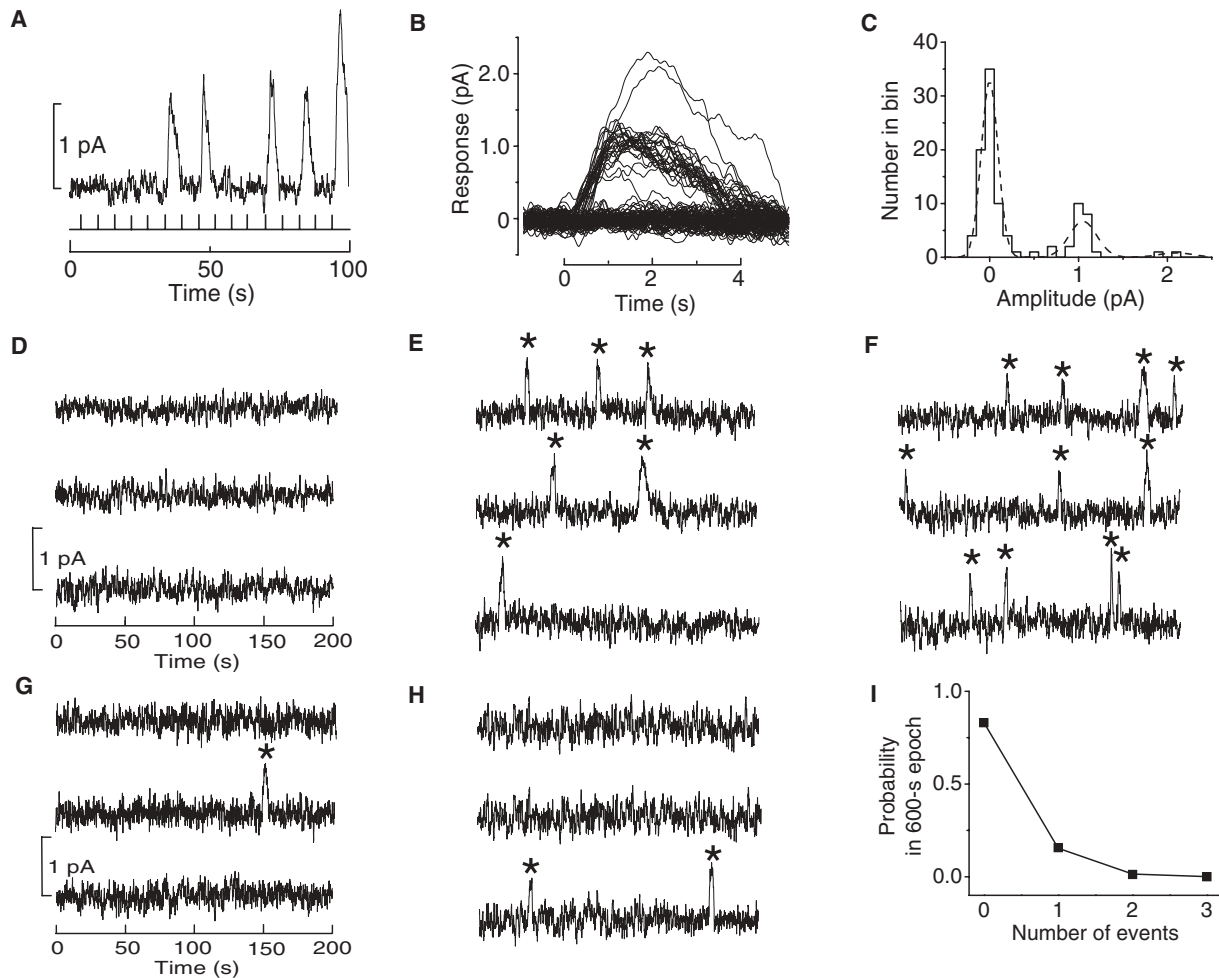
pigment. (Left) Flash responses of a transgenic mouse rod expressing S cone pigment but no rhodopsin. (Left inset) Corresponding normalized flash response-intensity relation, with the curve being  $R/R_{\max} = 1 - \exp(-I_f/K)$ , where  $R$  and  $R_{\max}$  are response and saturated response, respectively,  $I_f$  is flash intensity, and  $K$  is a sensitivity constant. Half-saturating flash intensity,  $\sigma$ , is 101 photons  $\mu\text{m}^{-2}$  (360 nm). (Middle) Temperature effect on spectrum. Same format as in (A), with temperatures at 37.5°C (red, 8 cells) and 25°C (black, 13 cells). (Right) Difference spectrum between the two temperatures, giving  $\lambda_{\max}/\lambda_c$  of 0.841.



given by  $A \times f_{\geq E_a^T}$ , where  $A$  is the preexponential factor (35) representing the frequency of spontaneous-activation attempts by the molecule. We began with  $A$  being the same for all pigments, but let the comparison with measurements indicate otherwise. Thus, the predicted thermal-rate ratio between two pigments is simply their  $f_{\geq E_a^T}$  ratio. Five pairwise comparisons were made, allowing us to: (i) examine the change in pigment noise due to an  $A_1/A_2$ -chromophore switch, (ii) compare rod and cone pigments containing the same chromophore, and (iii) cover pigments across the full visible spectrum. Consistent with Barlow's hypothesis (8), a longer  $\lambda_{\max}$  is indeed associated with a higher predicted noise rate constant (Table 1).

Considering that  $E_a^T < E_a^P$  is a more plausible situation (32–34), we repeated the above calculations with  $\alpha < 1$  (within a realistic range) in  $E_a^T = \alpha E_a^P$ —thus,  $m < 45$ —but we found the  $f_{\geq E_a^T}$  ratios to be hardly affected (table S2). We compared the above predictions ( $\alpha = 1$ ) with direct measurements, either previous measurements or new ones—all based on individually resolvable spontaneous events (measured electrophysiologically) for reliability (Table 1, but see also table S3). We repeated some measurements for consistency or for extrapolating them to 23°C (figs. S3 to S5) (18). We also measured the thermal noise of blue-cone pigment, naturally expressed in amphibian “green rods” besides blue cones (figs. S6 and S7) (18). Figure 3A shows sample re-

sponses of a *Bufo* green rod to repeated, identical dim flashes (440 nm), with the flash intensity sufficiently dim to elicit no response in most trials, thus allowing individual single-photon responses to be observed. The successful responses were quantized (Fig. 3B), with a unit amplitude of ~1 pA and an amplitude histogram matching the Poisson distribution as expected (Fig. 3C, dashed profile) (18). The green rod was rather quiet in darkness (Fig. 3D), but dim steady light elicited events similar to the single-photon responses (Fig. 3, E and F), indicating that the dark quiescence reflected low spontaneous activity instead of undetectably small events. In altogether 830 min of dark recordings from 42 cells, we observed only 15 events (Fig. 3, G and H) that



**Fig. 3.** Thermal pigment activity in blue-cone pigment (*Bufo* green rod). (A to C) Single-photon-response analysis. (A) Sample responses from 100 identical dim-flash trials (440 nm). Flash timings are indicated by vertical bars at bottom. (B) All 100 dim-flash responses superposed, showing quantized amplitudes. 10-msec flashes were centered at time zero. (C) Amplitude histogram from (B) measured at the transient peak of the averaged response. Bin-width is 0.1 pA. The dashed curve is a fit with the Poisson distribution blurred by Gaussian functions [eq. S3 in (18)], with a mean single-photon-response amplitude of 1.1 pA. (D) 10-min dark continuous recording from a different cell. (E and F) 10-min recordings from the same cell as in (D), with dim steady light of  $5.36 \times 10^{-4}$  and  $1.07 \times 10^{-3}$  photons  $\mu\text{m}^{-2} \text{s}^{-1}$  (440 nm), respec-

tively, expected to give 6 and 12 photoisomerization events, respectively, based on an effective collecting area (41) of  $19.1 \mu\text{m}^2$ , calculated from cell dimensions (table S5). These expected numbers closely match the 6 (E) and 11 (F) discrete events marked by stars. (G and H) 10-min dark recordings from two other cells showing one and two spontaneous events, respectively. The latter case is an extremely rare occurrence. (I) Poisson analysis of the dark spontaneous events collected from all cells (see text). The probability of zero, one, two, and three events observed, in a total of 83 trials of 10-min dark recording each, is plotted as the square symbols. The solid line shows the very good fit by the Poisson distribution (18) with a mean event rate of  $0.00031 \text{s}^{-1}$ . All recordings in this figure were low-pass filtered at 3 Hz.

likewise obeyed Poisson statistics over all cells (Fig. 3I) (18). These values gave an average spontaneous rate of  $0.00031\text{ s}^{-1}\text{ cell}^{-1}$ , which is literally at the lower limit of measurement. A previous estimate from salamander blue cones with indirect noise analysis only limited the rate to  $<2\text{ s}^{-1}\text{ cell}^{-1}$  (10), whereas an early report (36) on *Bufo* green rods inexplicably gave a rate of  $0.065\text{ s}^{-1}\text{ cell}^{-1}$ , a value that is 200-fold higher than what we report here (table S3). Correlating the rate trend with  $\lambda_{\text{max}}$ , we expect the mouse UV pigment to be too quiet for noise measurement, as appears to be the case (27).

From Table 1, the predicted rate ratio between  $A_1$  and  $A_2$  rhodopsins, as well as that between  $A_1$  and  $A_2$  red cone pigments, is either equal to or within a factor of 2 of the measured ratios. On the other hand, the predicted rate ratio between  $A_1$  rhodopsin and an  $A_1$  cone pigment, whether red or blue, underestimates the measured ratio by about one order of magnitude. The simplest explanation would be that the preexponential factor,  $A$ , is actually an order of magnitude higher for cone pigments, consistent with their more open chromophore-binding pocket (37–39). After adjusting for this difference (table S4), the remaining prediction/measurement discrepancy (~fivefold) in the comparison between  $A_2$  rhodopsin and  $A_2$  red cone pigment may stem from a measurement uncertainty and/or minor differences in, for example,  $m$  values across pigments. Overall, however,

the agreements are substantial. The comparison between  $A_1$  blue cone pigment ( $\lambda_{\text{max}} = 432\text{ nm}$ ) and  $A_2$  red cone pigment ( $\lambda_{\text{max}} = 617\text{ nm}$ ), which have the largest  $\lambda_{\text{max}}$  separation among non-UV visual pigments and cover a  $\sim 10^7$ -fold difference in rate constants, gives a mere 15-fold discrepancy between prediction and measurement. In contrast, the commonly used Boltzmann distribution gave predictions drastically different from measurements (fig. S8).

Our theory, developed to explain thermal activation of pigments, should also apply to photoactivation at  $\lambda > \lambda_c$ , where thermal energy contributes to photoisomerization. Interestingly, with  $A_1$  rhodopsin as an example, the spectral template (21) over an experimentally validated 10-log-unit descent at long wavelengths can be described by our theory (18), but requires a very small  $m$  value varying between 1 and 4 (Fig. 4A; see similar results for cone pigments in fig. S9). The large difference in  $m$  value between photoisomerization and thermal isomerization (nominally  $\sim 45$ , see above) probably reflects different molecular time windows in recruiting vibrational energy. In photoexcitation, only a few vibrational modes can be recruited, presumably due to instantaneous Franck-Condon excitation (35). Thermal activation, on the other hand, has an open time window, happening when, and only when, the requisite energy is recruited from a large number of collaborative vibrational modes. With the high  $E_a^T$

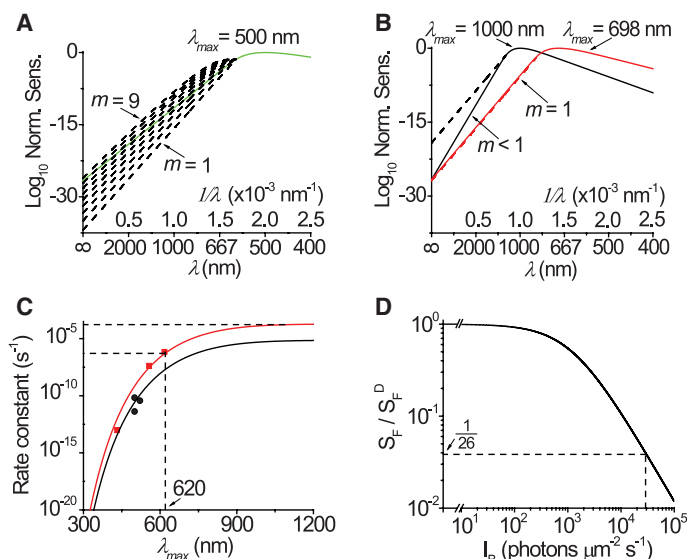
(ground-state isomerization energy barrier), thermal isomerization happens with an exceedingly low probability, thus explaining the low rate constants (Table 1).

The visual pigment with the longest  $\lambda_{\text{max}}$  known so far in nature is the  $A_2$  red cone pigment ( $\lambda_{\text{max}} \sim 620\text{ nm}$ ). Is there any physical or biological reason why pigments with longer  $\lambda_{\text{max}}$  values are evolutionarily disfavored? On the physical side, Fig. 4B shows a hypothetical  $A_1$  pigment template with  $\lambda_{\text{max}}$  at 698 nm, the long-wavelength descent of which matches predictions with  $m = 1$  (the Boltzmann limit) (18). Superficially, no pigment can have  $\lambda_{\text{max}} > 698\text{ nm}$  because no molecule, however small, could have  $m < 1$  (5). For example, a pigment with a hypothetical  $\lambda_{\text{max}}$  of 1000 nm would give  $m < 1$  (Fig. 4B). In reality, however,  $m$  is a nominal number (see earlier); thus,  $m < 1$  is possible provided that one or more vibrational modes contribute an energy less than the nominal value of  $kT$ . Thus, at least in principle,  $\lambda_{\text{max}} > 698\text{ nm}$  is still physically possible. How about biological considerations? In Fig. 4C, we extrapolate with our theory the measured noise rate constants to hypothetical pigments with  $\lambda_{\text{max}}$  values in the infrared. The rate constant for cone pigments approaches an asymptote of  $\sim 1.1 \times 10^{-4}\text{ s}^{-1}$  in the infrared, or 360 times higher than that of the 620-nm  $A_2$  red cone pigment. If a pigment with such an asymptotic rate constant were present in a salamander red cone

**Table 1.** Comparison between theoretical predictions and measurements of relative thermal-activation rate constants of visual pigments. For theoretical predictions, see text. For experimental measurements, *Bufo* red rods and green rods were at 23°C (this study), *Xenopus* rods [wild type (WT) and transgenics expressing human red cone pigment] were at 21° to 23°C (11), and mouse rods (WT and transgenics expressing human red cone pigment) were at 37°C (14) and 29°C (this study) and were extrapolated to 23°C, as described in (18) and fig. S4. This extrapolation is not perfect because of margins of error in the measured thermal rates. This issue, together with the approximations in cell dimensions (table S5) and  $\lambda_{\text{max}}$  determinations, may explain the order-of-magnitude discrepancy between the rate constants for *Bufo* red rod and mouse rod (both with  $A_1$ -rhodopsin) at 23°C. In principle, the two values would be expected to be identical according to our theory.

| Cell/pigment                                                   | $\lambda_{\text{max}}$<br>(nm) | $E_a^T$<br>(kcal mol <sup>-1</sup> ) | $f_{\geq E_a^T}$      | Predicted<br>rate-constant ratio | Measured rate<br>constant (s <sup>-1</sup> ) | Measured<br>rate-constant ratio |
|----------------------------------------------------------------|--------------------------------|--------------------------------------|-----------------------|----------------------------------|----------------------------------------------|---------------------------------|
| <i>Bufo</i> red rod/<br>$A_1$ <i>Bufo</i> rhodopsin            | 500                            | 48.03                                | $3.65 \times 10^{-6}$ | $\frac{1}{4.6}$                  | $4.18 \times 10^{-12}$                       | $\frac{1}{8.9}$                 |
| <i>Xenopus</i> rod/<br>$A_2$ <i>Xenopus</i> rhodopsin          | 521                            | 46.10                                | $1.67 \times 10^{-5}$ |                                  | $3.70 \times 10^{-11}$                       |                                 |
| Transgenic mouse rod/<br>$A_1$ human red cone pigment          | 557                            | 43.12                                | $1.52 \times 10^{-4}$ | $\frac{1}{16}$                   | $4.14 \times 10^{-8}$                        | $\frac{1}{16}$                  |
| Transgenic <i>Xenopus</i> rod/<br>$A_2$ human red cone pigment | 617                            | 38.93                                | $2.44 \times 10^{-3}$ |                                  | $6.70 \times 10^{-7}$                        |                                 |
| Mouse rod/<br>$A_1$ mouse rhodopsin                            | 500                            | 48.03                                | $3.65 \times 10^{-6}$ | $\frac{1}{42}$                   | $6.64 \times 10^{-11}$                       | $\frac{1}{623}$                 |
| Transgenic mouse rod/<br>$A_1$ human red cone pigment          | 557                            | 43.12                                | $1.52 \times 10^{-4}$ |                                  | $4.14 \times 10^{-8}$                        |                                 |
| <i>Xenopus</i> rod/<br>$A_2$ <i>Xenopus</i> rhodopsin          | 521                            | 46.10                                | $1.67 \times 10^{-5}$ | $\frac{1}{146}$                  | $3.70 \times 10^{-11}$                       | $\frac{1}{18,000}$              |
| Transgenic <i>Xenopus</i> rod/<br>$A_2$ human red cone pigment | 617                            | 38.93                                | $2.44 \times 10^{-3}$ |                                  | $6.70 \times 10^{-7}$                        |                                 |
| <i>Bufo</i> green rod/<br>$A_1$ <i>Bufo</i> blue cone pigment  | 432                            | 55.59                                | $5.17 \times 10^{-9}$ | $\frac{1}{706}$                  | $9.39 \times 10^{-14}$                       | $\frac{1}{45}$                  |
| <i>Bufo</i> red rod/<br>$A_1$ <i>Bufo</i> rhodopsin            | 500                            | 48.03                                | $3.65 \times 10^{-6}$ |                                  | $4.18 \times 10^{-12}$                       |                                 |

**Fig. 4.** Predictions from our theory. **(A and B)** Predictions of spectral descent at wavelengths longer than  $\lambda_c$ . **(A)** Spectral descent for  $A_1$  rhodopsin ( $\lambda_{\max} = 500$  nm) template matches predictions (dashed curves) drawn from eq. S7 (18) with  $m = 1$  through 9. **(B)** Spectral template for hypothetical  $A_1$  pigment with  $\lambda_{\max} = 698$  nm has a long-wavelength descent matching predictions throughout with  $m = 1$ , whereas that with  $\lambda_{\max} = 1000$  nm requires predictions with  $m < 1$ . Dashed curves (with one completely overshadowed by the red template) are predictions with  $m = 1$ . **(C and D)** Pigment noise prediction and its impact on photosensitivity. **(C)** Predicted thermal-noise rate constant as a function of  $\lambda_{\max}$  (data from Table 1). Black circles, rhodopsins; red squares, cone pigments. Curves are  $A \times f_{\geq E_1}$  at 23°C,  $m = 45$ , with  $A = 7.19 \times 10^{-6} \text{ s}^{-1}$  for rhodopsins and  $1.88 \times 10^{-4} \text{ s}^{-1}$  for cone pigments. **(D)** Effect of thermal activity on photosensitivity. The solid curve denotes the Weber-Fechner relation describing the reduction in flash sensitivity by background light,  $S_F/S_F^D = I_0/(I_0 + I_B)$ , where  $S_F$  and  $S_F^D$  are flash sensitivities in background light of intensity  $I_B$  and in darkness, respectively. The background intensity ( $I_0$ ) that reduces the sensitivity in darkness by half is 1200 isomerizations per second for salamander red cones (10), used here as a reference. The asymptotic thermal-noise rate for cone pigments from (C) is  $29,700 \text{ s}^{-1}$ , which would reduce light sensitivity of salamander red cones by 26-fold. In this estimate, we have ignored the relatively low  $\sim 200 \text{ s}^{-1}$  noise rate intrinsic to the salamander red cone.



[with  $\sim 2.7 \times 10^8$  pigment molecules (39)], the noise rate would be  $29,700 \text{ s}^{-1} \text{ cell}^{-1}$ , which would reduce the cell's already low sensitivity by another 26-fold, according to its adaptation behavior to background light (Fig. 4D) (10). Furthermore, the standard deviation of the background noise would increase by  $(360)^{1/2} = 19$ -fold. Such signaling detriments are undesirable. Perhaps for this reason, the viper pit organ detects infrared radiation with a heat-sensing ion channel rather than a visual pigment (40). For a short-wavelength-sensitive pigment, although its noise literally disappears at  $\lambda_{\max} < 400$  nm (Fig. 4C), nonspecific light absorption by proteins, peaking at  $\sim 280$  nm, becomes a limiting factor. These considerations probably explain, at least partially, why the  $\lambda_{\max}$  values of native visual pigments are confined to the narrow bandwidth of  $\sim 360$  to 620 nm, limiting color vision accordingly.

In summary, our work strongly suggests that thermal activation of visual pigments, like photoisomerization, involves a canonical isomerization reaction. If not for the discrepancy between the electrophysiological and photochemical measurements on rhodopsin, the inadequacy of Boltzmann statistics (i.e., involving only one vibrational mode) for understanding the thermal behavior of pigments would not have been obvious (for example, within a limited temperature range, Eq. 1 also gives an almost linear relation in an Arrhenius plot, as Boltzmann statistics does; see fig S5). Because

all biological molecules, like visual pigments, are polyatomic and thus have many vibrational modes, our success here hopefully will stimulate the same approach to other biomolecules.

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1307/DC1  
Materials and Methods  
Figs. S1 to S9  
Tables S1 to S5  
References

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# A DNA Damage Response Screen Identifies RHINO, a 9-1-1 and TopBP1 Interacting Protein Required for ATR Signaling

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The DNA damage response (DDR) is brought about by a protein kinase cascade that orchestrates DNA repair through transcriptional and posttranslational mechanisms. Cell cycle arrest is a hallmark of the DDR. We screened for cells that lacked damage-induced cell cycle arrest and uncovered a critical role for Fanconi anemia and homologous recombination proteins in ATR (ataxia telangiectasia and Rad3-related) signaling. Three DDR candidates, the RNA processing protein INTS7, the circadian transcription factor CLOCK, and a previously uncharacterized protein RHINO, were recruited to sites of DNA damage. RHINO independently bound the Rad9-Rad1-Hus1 complex (9-1-1) and the ATR activator TopBP1. RHINO was recruited to sites of DNA damage by the 9-1-1 complex to promote Chk1 activation. We suggest that RHINO functions together with the 9-1-1 complex and TopBP1 to fully activate ATR.

The importance of the DNA damage response (DDR) is underscored by the prevalence of mutations in this pathway found in cancers and developmental syndromes (*1*). Historically, most DDR genes were identified genetically in yeast as mutants defective in the transcriptional or cell cycle arrest responses to DNA damage. However, yeast lacks many mammalian DDR components. To identify mammalian DDR genes, we developed a high throughput microscopy-based assay using human osteosarcoma (U2OS) cells. Specific proteins were depleted from cells with small interfering RNA (siRNAs), and cells were monitored to measure inappropriate entry into mitosis 18 hours after exposure to 10 Gy of ionizing radiation (IR) (Fig. 1A). Most cells entering mitosis during this prolonged assay incurred damage during S phase [supporting online material (SOM) text and fig. S1]. Cell responses were stratified by the fold change in mitotic index (MI) compared with that of negative control cells: strong (>eight-fold), medium (four- to eight-fold), and weak (two- to four-fold) (Fig. 1B and table S1). Several known genes involved in checkpoint arrest, like Chk1, were not detected due to toxicity (fig. S2). We rescreened the toxic subset of genes at a lower siRNA concentration and detected an additional 98 genes scoring that included Chk1, PALB2, Wee1, and FANCM (fig. S2D and tables S1 and S2).

All strong and medium candidates and a subset of prioritized weak candidates, 720 in total,

selected for their degree of bypass or phosphorylation in response to DNA damage (*2, 3*) were chosen for secondary screening. Pools of siRNAs were separated into four individual siRNAs and retested. Effects of >75% of the pools recapitulated with at least one siRNA (Fig. 1C and table S3); 12% of these were eliminated because they increased MI in the absence of damage (Fig. 1C, fig. S3B, and table S3).

DDR mutations often cause sensitivity to DNA damage; therefore, sensitivity to mitomycin C (MMC) was assessed after depletion by siRNAs (fig. S3C). Of these, 53% that were detected with at least two siRNAs in the checkpoint assay also were detected with two or more siRNAs in the MMC-sensitivity assay (97 genes). These genes were further characterized with Dharmacon's On target plus siRNAs and were tested for checkpoint function, MMC-sensitivity, and homologous recombination (HR) efficiency (*4*) (fig. S4A and table S4; see SOM text for details). This high confidence list was enriched in genes annotated for the biological categories of DNA replication, recombination, and repair, as well as nucleic acid metabolism and cancer relevance (fig. S4B).

Bioinformatic analysis revealed a strong enrichment for the ATR [ataxia telangiectasia and Rad3-related], Fanconi anemia (FA), and HR pathways (Fig. 1D and fig. S4C). This seemed counterintuitive because double-strand breaks (DSBs) remain unrepaired in the absence of HR, and DDR signaling should persist until the repair process is complete. However, examination of Chk1 phosphorylation kinetics indicated that, in the absence of the breast cancer susceptibility proteins BRCA1 and BRCA2, ATR signaling was not sustained (Fig. 1, E and F). Although BRCA1 regulates Chk1 phosphorylation (*5*), we observed no defect in Chk1 activation 1 hour after exposure of cells to IR, but we observed a defect in maintenance of the activation (Fig. 1F). BRCA1 acts upstream of BRCA2, which has a

direct role in promoting Rad51 filament formation (*1*). Although HR is the major DSB repair pathway in S and G2 phases, other competing repair pathways such as nonhomologous end-joining (NHEJ) also contribute. To examine whether the removal of signaling DNA ends by NHEJ repair blocks HR in BRCA-depleted cells, we inhibited NHEJ by depleting 53BP1 with short hairpin RNA (shRNA) or with an inhibitor of DNA-dependent protein kinase catalytic subunit (NU7441). We chose 53BP1 because of its role in promoting ligation of DNA ends that are not in close proximity (*1*), a characteristic of ends that remain unrepaired for a long period of time. MI was decreased in BRCA1-depleted cells under both conditions (Fig. 1, G and H), suggesting that NHEJ processes the breaks and that this allows restart of the cell cycle. We did not observe a rescue of the BRCA2 defect, suggesting that either the processed DSB intermediates that accumulate in these cell lines are not repaired by NHEJ or that BRCA2 has an additional role in the DDR to promote arrest. Inhibiting NHEJ also suppressed the signaling defects in cells depleted of FANCM, FANCL, and FANCD1 (Fig. 1, G and H). These results are consistent with the finding that loss of 53BP1 partially rescues the HR defect and damage sensitivity of BRCA1 mutant cells, but not BRCA2 mutants, and of sensitivity to damage in FA-deficient cells (*6–9*).

Twenty-four genes were selected based on the secondary assays to assess their localization to sites of damage, a hallmark of DDR proteins (*10*). U2OS cells stably expressing green fluorescent protein (GFP) fusions were created and subjected to laser-induced DNA damage, marked by  $\gamma$ -H2AX costaining. Three proteins (INTS7, CLOCK, and MGC13204, an orphan protein) showed colocalization with  $\gamma$ -H2AX in the form of laser stripes (Fig. 2A) but did so independently of H2AX (fig. S5A).

CLOCK (checkpoint bypass with 7 of 8 siRNAs; MMC sensitivity with 6 of 8 siRNAs) is a basic helix-loop-helix transcription factor that participates in circadian rhythm regulation. Numerous examples of cross-talk between DNA damage, cell cycle regulation, and circadian rhythms have been described (*11*). Loss of CLOCK or its partner, BMAL1, in mice results in chronic enhanced sensitivity to DNA cross-linking agents, previously presumed to be due to misregulation of transcriptional targets (*12*). However, localization of CLOCK to sites of DNA damage suggests that it has a more direct role in the DDR.

INTS7 is a member of the Integrator complex that interacts with RNA polymerase II to assist in small nuclear RNA processing (*13*). Depletion of INTS7 resulted in bypass of cell cycle arrest (6 of 7 siRNAs) and sensitivity to MMC (3 of 7 siRNAs). One component of the Integrator complex, INTS3, associates with two related DNA single-strand binding proteins, SSB1 and SSB2, to form a complex recruited to damage sites and is required for checkpoint function (*14, 15*). INTS7 also interacted with SSB1 (fig. S5B).

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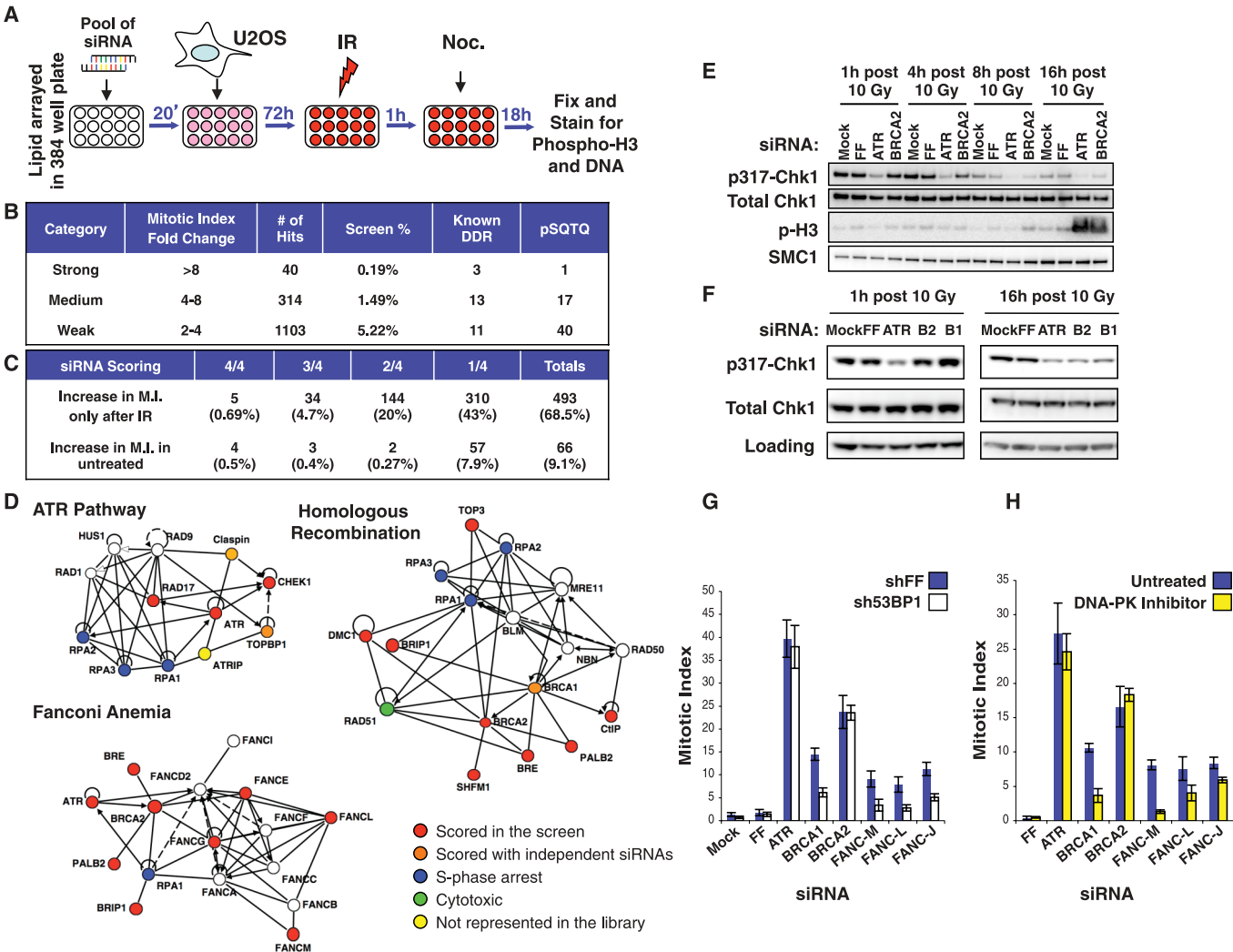
Depletion of MGC13204, referred to hereafter as RHINO, led to checkpoint bypass and sensitivity to MMC, IR, and camptothecin (Fig. 2D). Moreover, RHINO localized to foci in response to IR (fig. S6A), to MMC, and to ultraviolet (UV) laser stripes (Fig. 2A). The extent of RHINO depletion strongly correlated with both checkpoint and HR defects (Fig. 2, B and C, and figs. S6B and S8), which were suppressed by expression of an siRNA (#9)-resistant RHINO cDNA and partially suppressed by the wild-type (WT) cDNA (fig. S7).

Mass spectrometry analysis of proteins immunoprecipitated with Flag- hemagglutinin (HA)-tagged RHINO identified peptides for each subunit of the 9-1-1 complex (Rad9, Rad1, and

Hus1), which acts as a damage-specific clamp that binds the ATR activator TopBP1, the E3 ubiquitin ligase Rad18, and the E2 conjugating enzyme Ubc13 (Fig. 2E). Western blotting independently verified these interactions (Fig. 2F). To determine whether these components controlled RHINO's damage recruitment, we depleted TopBP1, Rad18, or Rad17 (which loads 9-1-1) and quantified RHINO localization to UV-induced DSBs. Rad17 depletion caused a 40% reduction in RHINO recruitment (fig. S9A). UV laser stripping analysis of WT and mutant mouse embryo fibroblasts (MEFs) expressing RHINO directly demonstrated the requirement of the 9-1-1 complex in RHINO recruitment (Fig. 3A and fig. S9B). For this reason, we named the orphan protein MGC13204 as

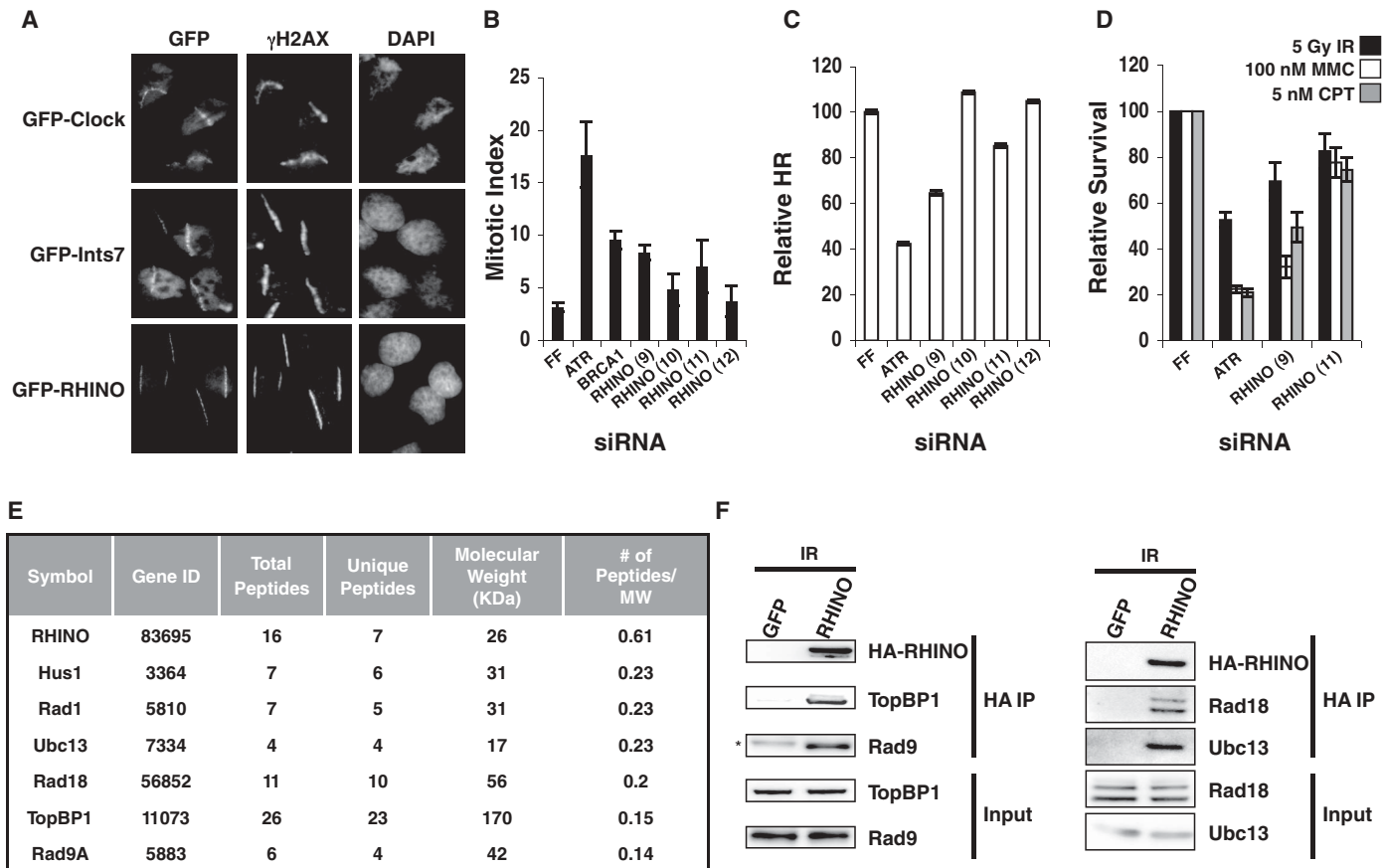
RHINO (Rad9, Rad1, Hus1 interacting nuclear orphan).

Rad18 and Ubc13 function in postreplication repair, which controls ubiquitination of proliferating cell nuclear antigen (PCNA) (16). Depletion of Rad18, but not RHINO, decreased UV-induced monoubiquitination of PCNA (fig. S10). Because TopBP1 and 9-1-1 have critical roles in ATR activation and phosphorylation of Chk1 (17), we investigated whether RHINO influenced IR-induced phosphorylation of Chk1. Indeed, depletion of RHINO led to a reduced phosphorylation of Chk1 (Fig. 3B) that was rescued by reexpression of RHINO (Fig. 3C). Together, these data suggest a role for RHINO in ATR-mediated activation of Chk1 driven by TopBP1 and 9-1-1.



**Fig. 1.** A screen for regulators of DDR signaling. **(A)** Schematic of the screen. **(B)** Primary screen statistics. The number of known DDR proteins and potential ataxia telangiectasia mutated (ATM)/ATR substrates are indicated under the header pSQTQ (phospho-SQTQ) for the conserved phosphorylation motif. **(C)** Secondary screen statistics for 720 candidate genes with and without DNA damage. For each gene, the fraction of siRNAs scoring and the total number of genes scoring is listed. **(D)** DDR networks identified in primary screen using Ingenuity pathway analysis. **(E)** ATR pathway signaling integrity after ATR and BRCA2 depletion. Cells collected at the indicated times after IR (10 Gy) were examined for phosphorylation of Chk1 on S317. SMC1 was used as loading

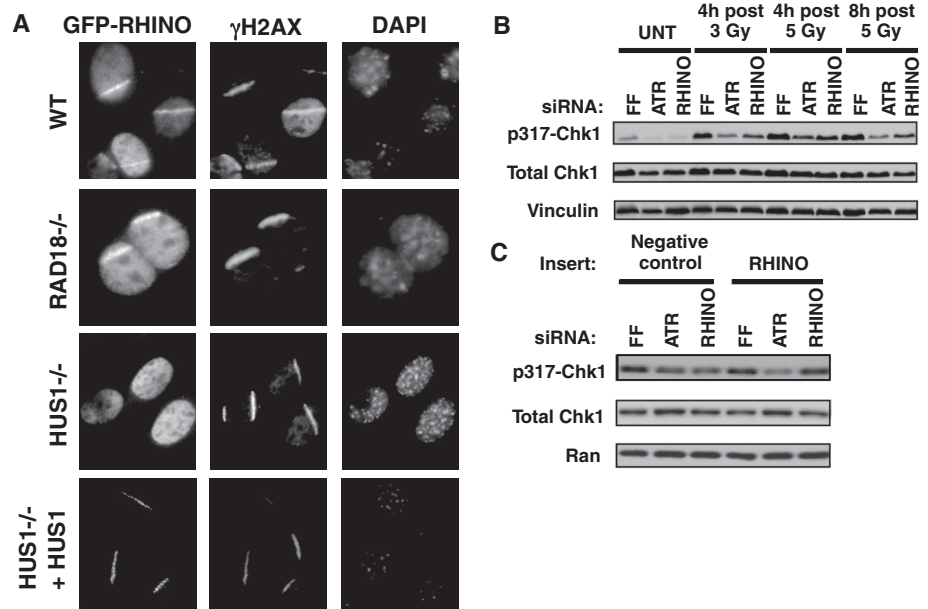
control. **(F)** ATR pathway signaling integrity after ATR, BRCA2 (B2), and BRCA1 (B1) depletion. Cells were collected 1 and 16 hours after IR (10 Gy). Cyclin B1 and PCNA were used as loading controls for the left and right panels, respectively. **(G)** Depletion of 53BP1 with shRNAs restores cell cycle arrest in BRCA1, FANCM-, FANCL-, and FANCLJ-depleted cells but not in ATR- or BRCA2-depleted cells. MI calculated 18 hours after 10 Gy. **(H)** Chemical inhibition of DNA-PKs restores cell cycle arrest in BRCA1-, FANCM-, FANCL-, and FANCLJ-depleted cells but not in ATR- or BRCA2-depleted cells. The DNA-PK inhibitor was added 2 hours after IR (10 Gy) at a final concentration of 1  $\mu$ M. MI was calculated as above.



**Fig. 2.** Characterization of several DDR candidates. **(A)** Analysis of DNA damage localization. U2OS cells expressing GFP fusions to INTS7, Clock, and RHINO were striped with a UV laser and costained with  $\gamma$ -H2AX to identify damaged cells. **(B)** Cell cycle arrest of RHINO with individual siRNAs at 30 nM, 18 hours after 10 Gy, is shown. siRNAs 9 and 11 result in statistically significant increases in MI with  $P$  values of  $8 \times 10^{-5}$  and 0.05, respectively, determined by a two-tailed  $t$  test. **(C)** HR results using individual RHINO siRNAs normalized to the negative control (FF). **(D)** The multicolor competition assay (MCA) was used to assess DNA damage

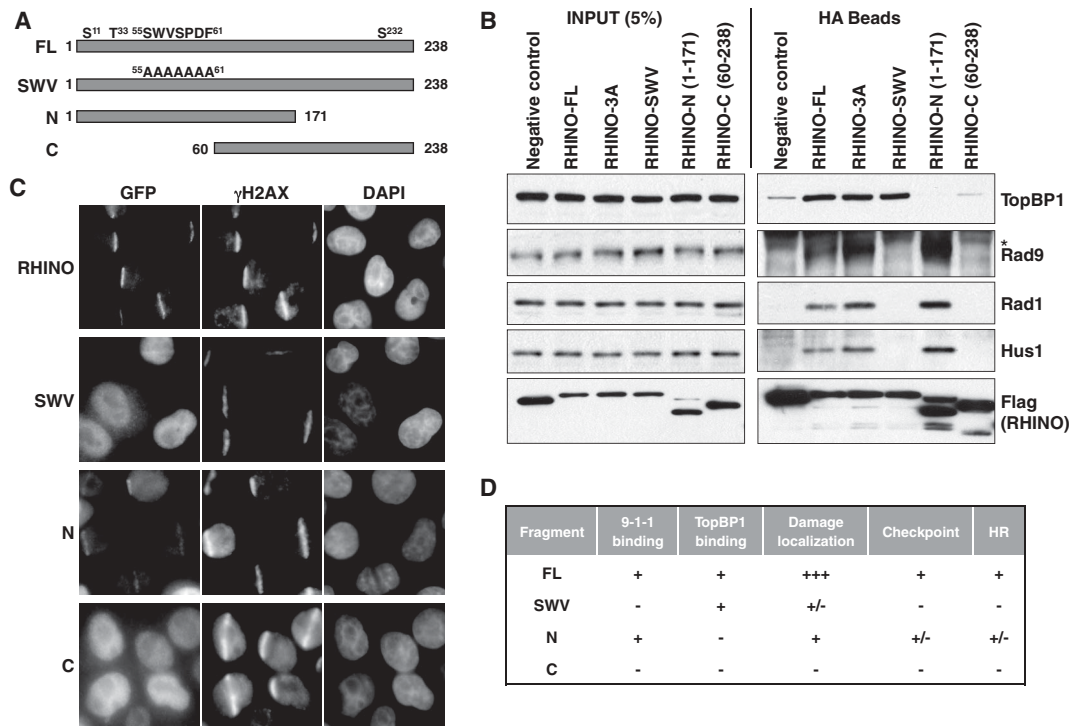
sensitivity of the indicated depleted genes in response to the indicated damaging agents. **(E)** Mass spectrometry analysis of Flag-HA RHINO after IR. Genes are ranked according to the ratio of the number of peptides versus the molecular weight of total protein. **(F)** Interaction of 9-1-1, TopBP1, Rad18, and Ubc13 with RHINO. Human embryonic kidney (293T) cells expressing GFP alone or Flag-HA-RHINO were treated with 10 Gy and harvested 4 hours later, and analyses for association by immunoblotting were carried out. The asterisk in the Rad9 panel represents the immunoglobulin G (IgG) heavy chain migrating above endogenous Rad9.

**Fig. 3.** Requirement of RHINO for DDR signaling. **(A)** Hus1-dependent RHINO recruitment to sites of DSBs. MEFs (WT, RAD18<sup>-/-</sup>, HUS1<sup>-/-</sup>, and HUS1<sup>-/-</sup> reconstituted with HUS1) expressing GFP-RHINO had DSBs introduced by UV laser treatment, and RHINO recruitment was assessed. **(B)** RHINO is required for Chk1 phosphorylation. Cells after ATR and RHINO depletion were treated with the indicated IR doses, collected at the indicated times, and immunoblotted. **(C)** Restoration of RHINO expression rescues DDR signaling. Three days after siRNA transfection, cells were infected at a multiplicity of infection of 2 with a retrovirus expressing the Flag-HA negative control or Flag-HA-RHINO. Eighteen hours later, cells were treated with 5 Gy and were harvested 4 hours later. Ran was used as a loading control.





**Fig. 4. RHINO interaction with 9-1-1 is critical for the DDR. (A)** Schematic representation of the different mutant and truncated versions of RHINO. **(B)** RHINO binds 9-1-1 and TopBP1 independently. Extracts from 293 T cells expressing either a Flag-HA negative control, Flag-HA-RHINO, or the indicated mutants were immunoprecipitated and immunoblotted with the indicated antibodies. The asterisk represents the IgG heavy chain. **(C)** RHINO localization requires 9-1-1. HeLa cells expressing WT GFP-RHINO or the indicated mutants were treated with the UV laser to introduce DSBs and assessed for RHINO localization. **(D)** Synopsis of the behavior of different mutants of RHINO.



RHINO orthologs exist in vertebrates, with high conservation in the N- and C-terminal regions (fig. S11A). The N terminus has similarity to the APSES (ASM-1, Phd1, StuA, EFG1, and Sok2) domain (18) (fig. S11B), a DNA binding domain homologous to the Kila-N domain found in eukaryotic viruses. To identify TopBP1 and 9-1-1 interaction regions, we mutated a conserved 7 amino acid region in the APSES domain [referred to as SWV for the first 3 amino acids of the motif (fig. S11)] and made C- and N-terminal truncations (Fig. 4A). The SWV mutation specifically blocked the interaction with the 9-1-1 complex but not TopBP1 (Fig. 4, B and D). Although the N-terminal, but not the C-terminal fragment bound 9-1-1, neither bound TopBP1 (Fig. 4, B and D). The SVW mutant was completely defective in localization to sites of damage, and the N-terminal fragment was partially impaired (Fig. 4, C and D, and fig. S12A), suggesting that the C-terminal region functions in localization to DNA damage. The interaction between RHINO and 9-1-1 is critical, because the SWV mutant RHINO failed to complement the checkpoint or HR defects (Fig. 4D and fig. S12). The RHINO N-terminal fragment partially rescued both phenotypes, consistent with its partial localization phenotype.

Depletion of Rad17, RHINO, or TopBP1 results in a partial defect in HR, although not as strong as that caused by ATR depletion (fig. S4A and S13). Depletion of both Rad17 and RHINO leads to a similar HR defect to that caused by individual depletion, suggesting that RHINO may mediate the 9-1-1's role in HR (fig. S13). Together, these data suggest that the 9-1-1/RHINO/TopBP1 axis promotes DDR activation in

response to lesions occurring during S phase (fig. S14).

We have uncovered a large number of genes and pathways required to maintain prolonged cell cycle arrest in response to DNA damage occurring in S phase. We uncovered an unexpected role for the HR and FA pathways in promoting cell cycle arrest. Although this may in part reflect differential funneling of repair intermediates into NHEJ, the inability of NHEJ inhibition to rescue the signaling defect from BRCA2 depletion suggests the possibility that cells may retain the capacity to signal if they process lesions through the HR pathway. There are two identified single-stranded DNA (ssDNA) binding complexes required for DDR signaling replication protein A (RPA) and the SSB1 and SSB2 complexes. The HR pathway, which replaces RPA with another ssDNA binding protein, Rad51, may represent a third (fig. S15 and SOM Text). We were unable to further test this possibility because of extreme toxicity of Rad51 depletion. However, it makes sense that cells would evolve the capacity to sense ongoing repair, because it is the completion of repair that is the relevant biological event with respect to turning off the DDR.

We also defined a new component of the Rad17/9-1-1/TopBP1 pathway, RHINO. TopBP1 recruitment to sites of damage through the Rad17/9-1-1 complex is important for activation of ATR (19). This recruitment has been suggested to occur through association of the BRCT repeats of TopBP1 with constitutively phosphorylated Rad9 (20). However, experiments in *Xenopus* have indicated that there is a 9-1-1-dependent but Rad9-phosphorylation-independent mechanism that also operates to recruit TopBP1 (21).

The association of RHINO with both 9-1-1 and TopBP1 through independent domains on RHINO suggests that it may bridge these to help recruit TopBP1 to the 9-1-1 complex. Whether RHINO is involved in this second mechanism, and why two different mechanisms operate, remains to be determined, but it is possible that they are each used in response to different classes of lesions or to bolster long-term signaling. Alternatively, RHINO could allosterically regulate TopBP1 to promote its association with, or activation of, ATR/ATR interacting protein (ATRIP).

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1313/DC1  
Materials and Methods

SOM Text  
Figs. S1 to S15  
Tables S1 to S4  
References

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# The mTOR-Regulated Phosphoproteome Reveals a Mechanism of mTORC1-Mediated Inhibition of Growth Factor Signaling

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The mammalian target of rapamycin (mTOR) protein kinase is a master growth promoter that nucleates two complexes, mTORC1 and mTORC2. Despite the diverse processes controlled by mTOR, few substrates are known. We defined the mTOR-regulated phosphoproteome by quantitative mass spectrometry and characterized the primary sequence motif specificity of mTOR using positional scanning peptide libraries. We found that the phosphorylation response to insulin is largely mTOR dependent and that mTOR exhibits a unique preference for proline, hydrophobic, and aromatic residues at the +1 position. The adaptor protein Grb10 was identified as an mTORC1 substrate that mediates the inhibition of phosphoinositide 3-kinase typical of cells lacking tuberous sclerosis complex 2 (TSC2), a tumor suppressor and negative regulator of mTORC1. Our work clarifies how mTORC1 inhibits growth factor signaling and opens new areas of investigation in mTOR biology.

The serine-threonine kinase mammalian target of rapamycin (mTOR) is a major controller of growth that is deregulated in cancer and diabetes (1, 2). mTOR is the catalytic subunit of two multiprotein complexes, mTORC1 and mTORC2. mTORC1 is activated by growth factors and nutrients through a pathway that involves the tuberous sclerosis complex (TSC1-TSC2) tumor suppressors as well as the Rag and Rheb guanosine triphosphatases (GTPases). mTORC1 phosphorylates the translational regulators S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E (eIF-4E) binding proteins (4E-BP1 and 4E-BP2), whereas mTORC2 activates Akt and serum- and glucocorticoid-regulated kinase 1 (SGK1) and is part of the growth factor-stimulated phosphoinositide 3-kinase

(PI3K) pathway. Collectively, mTORC1 and mTORC2 regulate processes that control cell growth and proliferation, including protein synthesis, autophagy, and metabolism. mTOR inhibitors derived from rapamycin, an allosteric mTORC1 inhibitor, have been in trials for anti-cancer uses, but the feedback activation of the PI3K-Akt pathway that occurs with mTORC1 inhibition may lessen their clinical efficacy (3).

The few mTOR substrates with defined phosphorylation sites likely cannot explain all processes under the control of mTOR (1, 2) (table S1). To discover additional substrates, we conducted a systematic investigation of the mTOR-regulated phosphoproteome using mass spectrometry and isobaric tags that permit four-way multiplexed relative and absolute quantification of phosphopeptide abundances (iTRAQ) (4). With duplicate analyses for each, we analyzed phosphopeptides from two sets of cells in which the pathway was hyperactivated and then inhibited with Torin1, a recently developed adenosine 5'-triphosphate (ATP)-competitive mTOR kinase domain inhibitor that blocks all known phosphorylations downstream of mTORC1 and mTORC2 (5). Human embryonic kidney (HEK)-293E cells were deprived of serum and then stimulated with insulin in the presence or absence of rapamycin or Torin1 (Fig. 1A). Wild-type (TSC2<sup>+/+</sup>) and TSC2-null (TSC2<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs), which have increased mTORC1 signaling, were also treated with or without Torin1 (Fig. 1A).

Under these conditions, phosphorylation events known to be downstream of mTORC1 (e.g., rapamycin-sensitive S6K1 T389 and rapamycin-insensitive 4E-BP1 T37 and T46) and mTORC2 (e.g., Akt S473, PRAS40/AKT1S1 T246, NDRG1 T346) behaved as expected (fig. S1).

From the HEK-293E cells, we identified 4256 unique phosphopeptides corresponding to 47 phosphotyrosine and 4204 phosphoserine-threonine sites on 1661 distinct proteins [false discovery rate (FDR) ~1%, table S2]. Using a cutoff of 2.5 median absolute deviations (MADs) below the median log<sub>2</sub>(Torin1/Insulin ratio) (robust z-score < -2.5), we identified 127 phosphopeptides from 93 proteins as sensitive to Torin1 and designated them as mTOR-regulated (Fig. 1B). From the MEFs, 7299 unique phosphopeptides corresponding to 110 phosphotyrosine and 7145 phosphoserine-threonine sites on 2406 distinct proteins were identified (FDR ~1%, table S2), of which 231 phosphopeptides from 174 proteins were regulated by mTOR [-2.5 MAD, log<sub>2</sub>(TSC2<sup>-/-</sup>Torin1/TSC2<sup>-/-</sup> vehicle)] (Fig. 1C). By this -2.5 MAD cutoff for both the HEK-293E and MEF data sets, the mTOR-regulated sites were highly enriched in canonical mTOR pathway phosphorylations (Fisher's exact test  $P = 5.2 \times 10^{-24}$  and  $6.5 \times 10^{-23}$ , respectively; Fig. 1, B and C, and table S1), an indication of the predictive potential of the data to identify mTOR pathway components. Additionally, we identified Torin1-sensitive sites on lesser or only recently characterized mTOR substrates [CAP-GLY domain containing linker protein 1 (CLIP1) S1158 (6), Unc-51-like kinase 1 (ULK1) S638 (7-9), and insulin receptor substrate 2 (IRS2) S616 (10)].

Global comparisons of the data sets revealed several interesting features. In the HEK-293E cells, phosphorylation changes resulting from Torin1 treatment were markedly similar to those observed under serum deprivation (Spearman's  $\rho = 0.66$ ,  $P \sim 0$ , Fig. 1D), revealing that insulin-regulated phosphorylations (both down- and up-regulated) are largely mTOR dependent. The effects of rapamycin and Torin1 treatment were similar (Spearman's  $\rho = 0.48$ ,  $P \sim 0$ , Fig. 1E), but a subset of Torin1-sensitive sites were not rapamycin sensitive (upper left quadrant, Fig. 1E), including 4E-BP1 and 4E-BP2 T37 and T46 (5, 11, 12) and the mTORC2-mediated Akt3 S472 and NDRG1 S330. Analysis of the MEF data set revealed that phosphorylations that increase with TSC2 loss are more likely to be inhibited by Torin1 (Spearman's  $\rho = -0.25$ ,  $P = 1.4 \times 10^{-130}$ ) (Fig. 1F). Hierarchical clustering of the conditions and sorting of the phosphopeptide abundances in the HEK-293E

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cells also verified the similarity between serum starvation and Torin1 treatment (fig. S2) and our ability to discriminate between known rapamycin-sensitive (fig. S2, top) and -insensitive (fig. S2, bottom) sites, and showed that phosphorylations that are rapamycin sensitive tend to be inhibited by Torin1 treatment to a greater extent than those that are not (fig. S2).

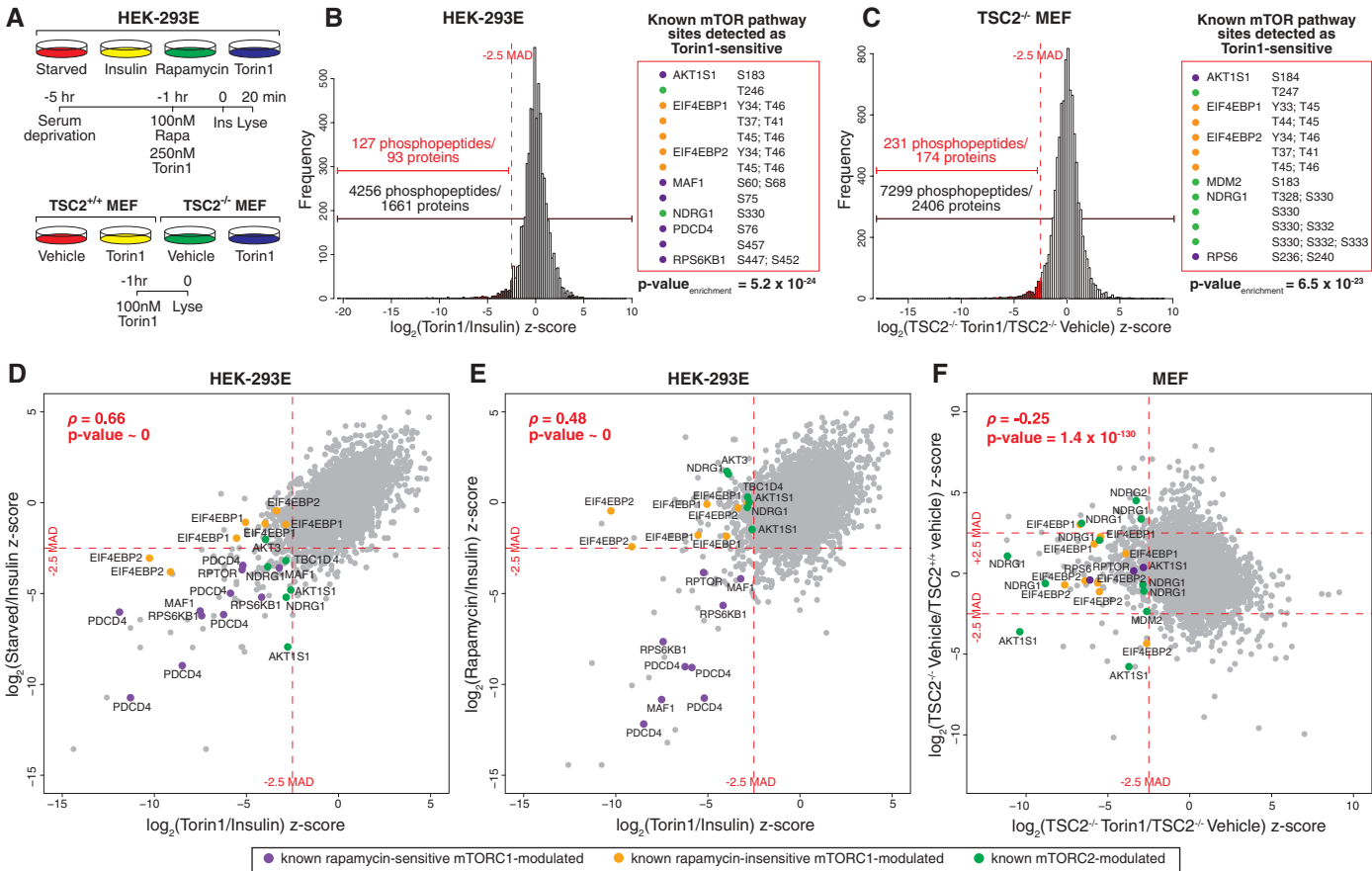
Pathway analysis of the candidate mTOR-regulated proteins revealed enrichment (FDR < 10%) in processes downstream of mTOR, such as translation [Gene Ontology (GO):0006417] and regulation of cell size (GO:0008361), as well as some not generally considered to be under mTOR control (table S3). These include RNA splicing (GO:0008380), DNA replication (GO:0006260), vesicle-mediated transport (GO:0016192), and regulation of mRNA-processing bodies (GO:0000932), signifying a broader role for mTOR signaling than presently appreciated.

As the mTOR-regulated sites may be phosphorylated by mTOR or by downstream kinases,

we sought to distinguish direct substrates from indirect effectors by determining a consensus phospho-acceptor motif for mTOR. An example of such a motif is the (R/K)X(R/K)XX(S\*/T\*) sequence [X, any amino acid; asterisk (\*), phospho-acceptor] recognized by the mTOR substrates Akt, S6K1, and SGK1, all members of the AGC kinase family (13). Because mTOR phosphorylates hydrophobic motifs (HMs) of the AGC kinases as well as the distinct proline-directed sites of proteins such as 4E-BP1 and 4E-BP2 (fig. S3), it is unknown if the kinase exhibits any motif specificity or if the choice of sites is entirely determined by factors beyond the primary substrate sequence. We found that when combined with its activator, guanosine 5'-triphosphate (GTP)-bound Rheb, highly pure and intact mTORC1 (14) robustly phosphorylated an arrayed positional scanning peptide library (15) (fig. S4 and Fig. 2A). Although mTORC1 and mTORC2 phosphorylate distinct sets of substrates, they likely have similar motif preferences as they share the same

catalytic domain. This unbiased assay revealed that mTOR possesses selectivity toward peptide substrates concordant with known mTOR sites (figs. S3 and S4 and Fig. 2, A and B), primarily at the +1 position at which mTOR prefers proline, hydrophobic residues (L, V), and aromatic residues (F, W, Y). This pattern of specificity at the +1 position is unique among all kinases previously profiled (16). mTOR also exhibits minor selectivity at other positions (fig. S4 and Fig. 2, A and B). These data suggest that within the HM of the AGC kinases (fig. S3) the -4 and -1 hydrophobic residues are dispensable for mTOR recognition.

Combining our two approaches, we classified the mTOR-regulated phosphorylation sites, first by rapamycin sensitivity [HEK-293E, -2.5 MAD  $\log_2$ (Rapamycin/Insulin)] or by increased phosphorylation in cells lacking TSC2 [MEFs, +2.5 MAD  $\log_2$ (TSC2<sup>-/-</sup> vehicle/ TSC2<sup>+/+</sup> vehicle)] (Fig. 2, C and D; figs. S5 and S6; and table S4). Rapamycin-sensitive sites or those up-regulated in TSC2<sup>-/-</sup>



**Fig. 1.** Identification of the mTOR-regulated phosphoproteome. (A) Phosphopeptide abundances were determined from two sets of samples: HEK-293E cells serum starved for 4 hours, treated with 100 nM rapamycin, 250 nM Torin1, or vehicle control for 1 hour, and then stimulated with 150 nM insulin for 20 min and TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup> MEFs treated with 100 nM Torin1 or vehicle control for 1 hour. (B and C) Distributions of robust z-scores [median absolute deviations (MADs) away from the median (B)  $\log_2$ (Torin1/Insulin) for HEK-293Es or (C)  $\log_2$ (TSC2<sup>-/-</sup> Torin1/TSC2<sup>-/-</sup> vehicle) for MEFs] for both replicates. P values associated with enrichment for known mTOR-modulated

sites among the -2.5 MAD Torin1-sensitive phosphopeptides were determined by Fisher's exact test. Phosphopeptides detected in both replicates had to meet the -2.5 MAD threshold both times to be considered mTOR-regulated. (D to F) Correspondence between (D) Torin1 treatment and serum deprivation in HEK-293Es, (E) Torin1 and rapamycin treatment in HEK-293Es, and (F) Torin1 treatment and up-regulation in TSC2<sup>-/-</sup> MEFs. The relevant robust z-scores for both replicates, phosphopeptides corresponding to known mTOR-modulated sites, Spearman's rank correlation coefficient ( $\rho$ ), and associated P values are indicated. Axes were truncated to aid in visualization.



cells are likely mTORC1 regulated, whereas the remaining sites could be downstream of either complex. Second, we scored the sites by motif into the following categories: (i) candidate direct mTOR sites, (ii) candidate AGC kinase substrates, or (iii) mTOR-regulated but by an undetermined mechanism (Fig. 2, C and D; figs. S5 and S6; and table S4).

Several candidate substrates implicate mTOR in new aspects of cell growth regulation. WD repeat domain, phosphoinositide interacting 2 (WIPI2) (fig. S6), a sparsely characterized ortholog of the yeast Atg18p, is a potential substrate implicated in autophagosome formation (17). In addition, orthologs of the candidate substrates protein associated with topoisomerase II homolog 1 (PATL1) (fig. S5 and S6) and La ribonucleoprotein domain family member 1 (LARP1) (figs. S5 and S6) bind RNA, localize to P-bodies, and control mRNA stability (18, 19). In yeast, Pat1p phosphorylation is sensitive to rapamycin (20), and contributes to repression of mRNA translation upon amino acid withdrawal (21), suggesting that regulation of mRNA degradation may be important for growth control. Other potential substrates point to nascent areas of mTOR biology. For example, mTOR putatively regulates the neural stem cell marker Nestin, the pleiotropic transcription factor c-Jun, and the myogenic stem cell transcription factor forkhead box K1 (FoxK1) (fig. S6).

One candidate of special interest was the adaptor protein growth factor receptor-bound

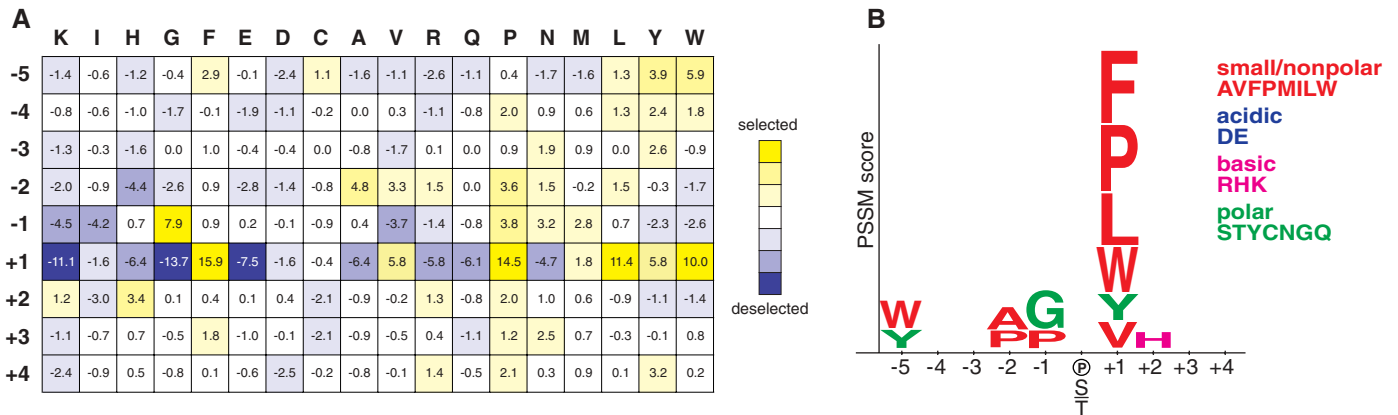
protein 10 (Grb10) (Fig. 2D and fig. S6). The abundance of a Grb10 phosphopeptide with putative mTOR motif sites was increased in the absence of TSC2 and decreased after Torin1 treatment in both TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup> MEFs (tables S2 and S4, Fig. 2D, and fig. S6), patterns consistent with being in the mTORC1 pathway. Conserved among vertebrates, Grb10 negatively regulates growth factor signaling (22). It binds the insulin and insulin-like growth factor 1 (IGF-1) receptors, and mice without Grb10 are larger and exhibit enhanced insulin sensitivity (23–25). Although the ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4 (Nedd4) does not directly ubiquitinate Grb10 (26), Nedd4-null mice have more Grb10 protein and are insulin and IGF resistant, a signaling phenotype reminiscent of cells lacking TSC1 or TSC2 (27). Therefore, we speculated that Grb10 might function downstream of mTORC1 to inhibit PI3K-Akt signaling.

In SDS-polyacrylamide gel electrophoresis analyses, Grb10 exhibited an insulin-stimulated mobility shift that is partially sensitive to rapamycin (Fig. 3A). In vitro phosphatase treatment eliminated the shift, as did Torin1, indicating that the shift results from phosphorylation and is dependent on mTOR activity (Fig. 3, A and B). Amino acids stimulated Grb10 phosphorylation and were required for its serum-dependent phosphorylation (Fig. 3C), and in TSC2<sup>-/-</sup> MEFs, Grb10 phosphorylation was retained in the ab-

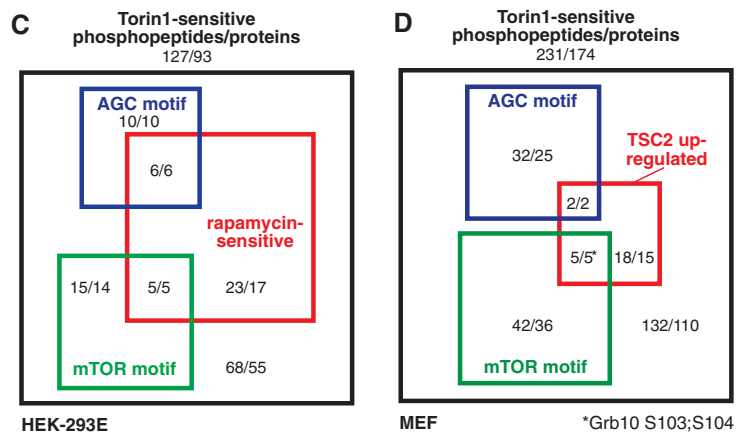
sence of serum but lost upon acute rapamycin and Torin1 treatment (Fig. 3D). These data point to mTORC1, but not mTORC2, as the main regulator of Grb10. Consistent with this conclusion, the loss of rictor, a core component of mTORC2, did not affect Grb10 phosphorylation (fig. S7, A and B).

In cells lacking S6K1 and S6K2, Grb10 was still regulated in an mTOR-dependent manner (fig. S7C), suggesting that it might be a direct substrate. Indeed, Grb10 was phosphorylated in vitro by mTORC1 to an extent comparable with that of known substrates (Fig. 3E). The sites regulated by mTOR in vitro (Fig. 3G) and in cells (Fig. 3H) were mapped to S104, S150, T155, S428, and S476, which are located in or near the proline-rich region or between the pleckstrin homology (PH) and Src homology 2 (SH2) domains (BPS) of Grb10 (Fig. 3F). In cells, all sites were Torin1 sensitive, while S476 was also rapamycin sensitive (Fig. 3H). Grb10 is therefore similar to 4E-BP1, an mTORC1 substrate with both rapamycin-sensitive and -insensitive sites (Fig. 3I). We verified our characterization of these sites with phosphospecific antibodies against S150, S428, and S476 (Fig. 3J and fig. S8, A and B). Mutation of the identified sites along with a few neighboring residues eliminated the mobility shift (Fig. 3K), indicating that most if not all mTOR-regulated sites were localized.

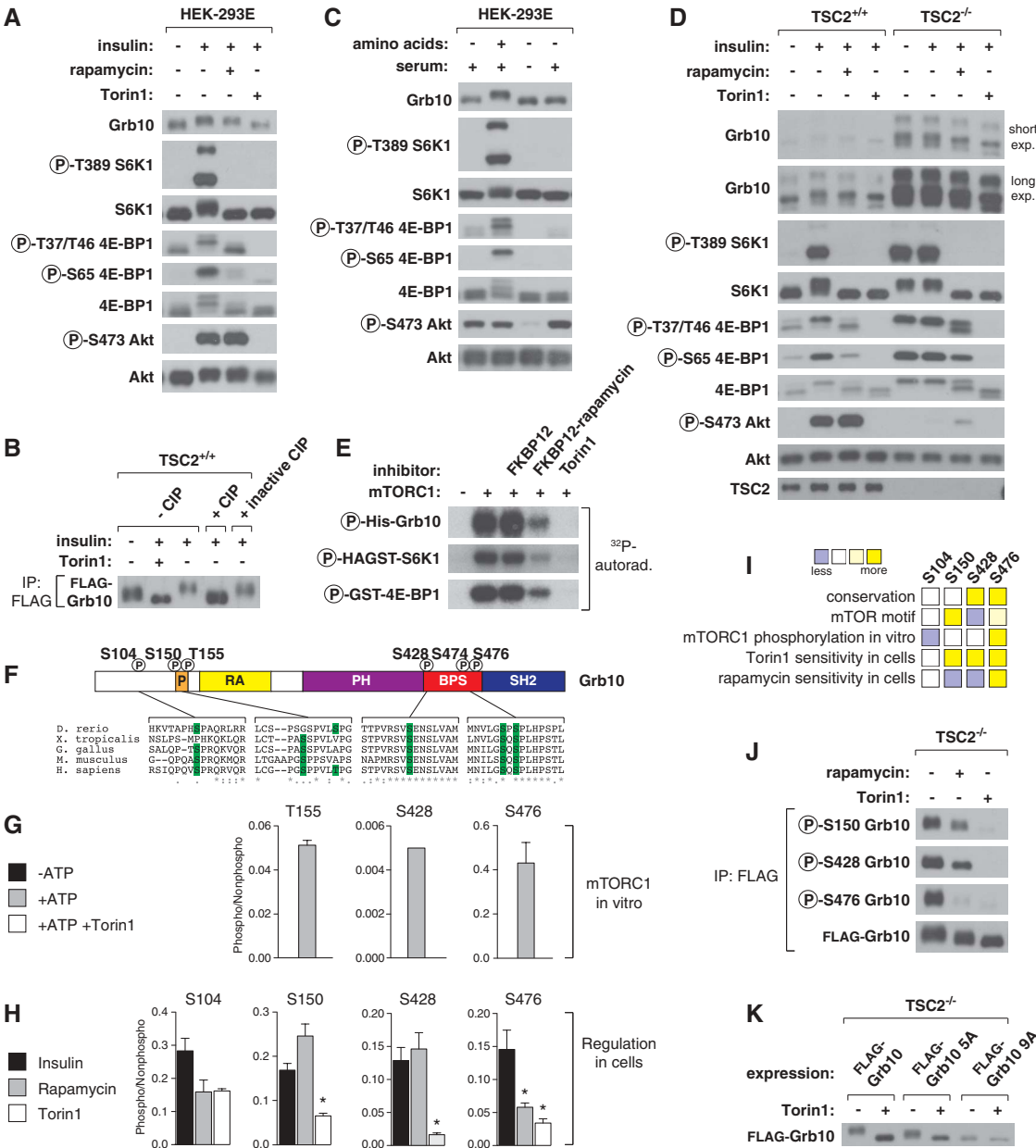
mTORC1 inhibits PI3K-Akt signaling, but the molecular connections involved are poorly



**Fig. 2.** Characterization of a consensus mTOR phosphorylation motif. **(A)** The position-specific scoring matrix (PSSM) resulting from quantification of the in vitro phosphorylation of a positional scanning peptide library by mTORC1. Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. **(B)** The visualized mTOR consensus motif. Letter height is proportional to the PSSM score. Only those selected residues with scores greater than a standard deviation from the average PSSM score within a row are shown. **(C and D)** Classification of the mTOR-regulated phosphopeptides in **(C)** HEK-293E and **(D)** MEFs organized by rapamycin sensitivity [ $-2.5 \text{ MAD} (\log_2 (\text{Rapamycin/Insulin}))$ ] or TSC2 up-regulation [ $+2.5 \text{ MAD} (\log_2 (\text{TSC2}^{-/-} \text{ vehicle/TSC2}^{+/+} \text{ vehicle}))$ ], consistency with the mTOR motif (fifth percentile by Scansite), or presence of an AGC motif [(R/K)X(R/K)XX(S\*/T\*)]. The numbers represent the number of unique phosphopeptides or proteins. Refer to figs. S5 and S6 and table S4 for more details.



understood. One mechanism is the destabilization of insulin receptor substrate 1 (IRS1) by S6K1 phosphorylation (10, 28). However, other mechanisms likely exist because loss of raptor, an essential mTORC1 component, in S6K1<sup>-/-</sup>S6K2<sup>-/-</sup> cells still activated Akt phosphorylation without affecting IRS1 abundance (Fig. 4A). Therefore, we tested whether mTORC1 might also inhibit the PI3K pathway through Grb10. Con-



**Fig. 3.** Grb10 is an mTORC1 substrate with rapamycin-sensitive and -insensitive sites. (A) HEK-293E cells were deprived of serum for 4 hours, treated with 100 nM rapamycin or 250 nM Torin1 for 1 hour, and then stimulated with 150 nM insulin for 15 min. Cell lysates were analyzed by immunoblotting. (B) TSC2<sup>+/+</sup> MEFs stably expressing FLAG-Grb10 were serum deprived for 4 hours, treated with 250 nM Torin1 for 1 hour, and then stimulated with 150 nM insulin for 15 min. All FLAG-tagged Grb10 constructs correspond to isoform c of human Grb10. FLAG immunoprecipitates were incubated in buffer, calf intestinal phosphatase (CIP), or inactivated CIP and analyzed by immunoblotting. (C) HEK-293E cells were deprived of amino acids or both amino acids and serum for 50 min, and then stimulated with either amino acids or serum for 10 min and analyzed by immunoblotting. (D) TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup> MEFs were treated and analyzed as in (A). (E) mTORC1 in vitro kinase assays with substrates in the presence of the indicated inhibitors and radiolabeled ATP were analyzed by autoradiography. (F) Schematic representation of Grb10 protein structure with the phosphorylation sites from vertebrate orthologs aligned below. Numbering is according to human isoform a. (G) The phosphorylation state of Grb10 from kinase assays performed similarly to (E) were analyzed by targeted mass spectrometry and phosphorylation ratios determined from chromatographic peak intensities. (H) FLAG immunoprecipitates from HEK-293E cells stably expressing FLAG-Grb10 treated as in (A) were analyzed as in (G). Data are means ± SEM (n = 2 to 6). \*P < 0.05 for differences between stimulated and treated conditions (Mann-Whitney *t* test). (I) A summary of (F), (G), and (H) for each Grb10 phosphorylation site. (J) FLAG immunoprecipitates from TSC2<sup>-/-</sup> MEFs stably expressing FLAG-Grb10 treated with 100 nM rapamycin or 250 nM Torin1 for 1 hour were analyzed by immunoblotting with Grb10 phospho-specific antibodies. (K) TSC2<sup>-/-</sup> MEFs stably expressing FLAG-Grb10, 5A (S150A T155A S158A S474A S476A), or 9A (S104A S426A S428A S431A) mutants treated with 250 nM Torin1 for 1 hour were analyzed by immunoblotting.





proteins, which are likely the most important effects of mTOR inhibitors to consider in their clinical use (Fig. 4G). Our findings also support the idea (30, 31) that concomitant IGF-1 receptor inhibition may improve the anticancer efficacy of mTOR inhibitors. Finally, the discovery of Grb10 as an mTORC1 substrate validates our approach and suggests that the other potential downstream effectors that we identified may also serve as starting points for new areas of investigation in mTOR biology.

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## Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1317/DC1  
Materials and Methods  
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# Phosphoproteomic Analysis Identifies Grb10 as an mTORC1 Substrate That Negatively Regulates Insulin Signaling

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The evolutionarily conserved serine-threonine kinase mammalian target of rapamycin (mTOR) plays a critical role in regulating many pathophysiological processes. Functional characterization of the mTOR signaling pathways, however, has been hampered by the paucity of known substrates. We used large-scale quantitative phosphoproteomics experiments to define the signaling networks downstream of mTORC1 and mTORC2. Characterization of one mTORC1 substrate, the growth factor receptor-bound protein 10 (Grb10), showed that mTORC1-mediated phosphorylation stabilized Grb10, leading to feedback inhibition of the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated, mitogen-activated protein kinase (ERK-MAPK) pathways. Grb10 expression is frequently down-regulated in various cancers, and loss of Grb10 and loss of the well-established tumor suppressor phosphatase PTEN appear to be mutually exclusive events, suggesting that Grb10 might be a tumor suppressor regulated by mTORC1.

The evolutionarily conserved Ser-Thr protein kinase mammalian target of rapamycin (mTOR) functions as the core catalytic component of two structurally and functionally distinct signaling complexes. mTOR complex 1 (mTORC1) regulates protein translation, autophagy, and cell growth, whereas mTOR complex 2 (mTORC2) regulates the actin cytoskeleton and cell survival (1–3). mTORC1 and mTORC2 respond to upstream inputs such as growth factors, energetic status, and amino acid levels (3),

but relatively few downstream targets of mTOR have been identified.

Misregulated mTOR activity is a common feature of most cancers (1), but clinical trials evaluating the mTORC1 selective inhibitor rapamycin as an anticancer agent have met with limited success (2). Rapamycin resistance has emerged as a major challenge to its clinical use (4) and is caused in part by feedback loops that activate the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated, mitogen-activated protein kinase

(ERK-MAPK) signaling pathways in rapamycin-treated cells through poorly understood mechanisms (5, 6). Identifying substrates of mTORC1 and mTORC2 will be important for understanding how mTOR signals downstream and for defining components of feedback loops involved in rapamycin resistance.

We performed two sets of large-scale, quantitative phosphoproteomics experiments to characterize the signaling network downstream of mTOR (Fig. 1 and figs. S1 to S3). The first stable isotope labeling with amino acids in cell culture (SILAC) experiment (Rapa screen) was performed using *Tsc2*<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) [see supporting online material (SOM) text for detailed description of the screen]. We identified 4484 and 6832 unique phosphorylation sites on 1615 and 1866 proteins from two biological replicate experiments, respectively (table S1 and databases S1 and S2).

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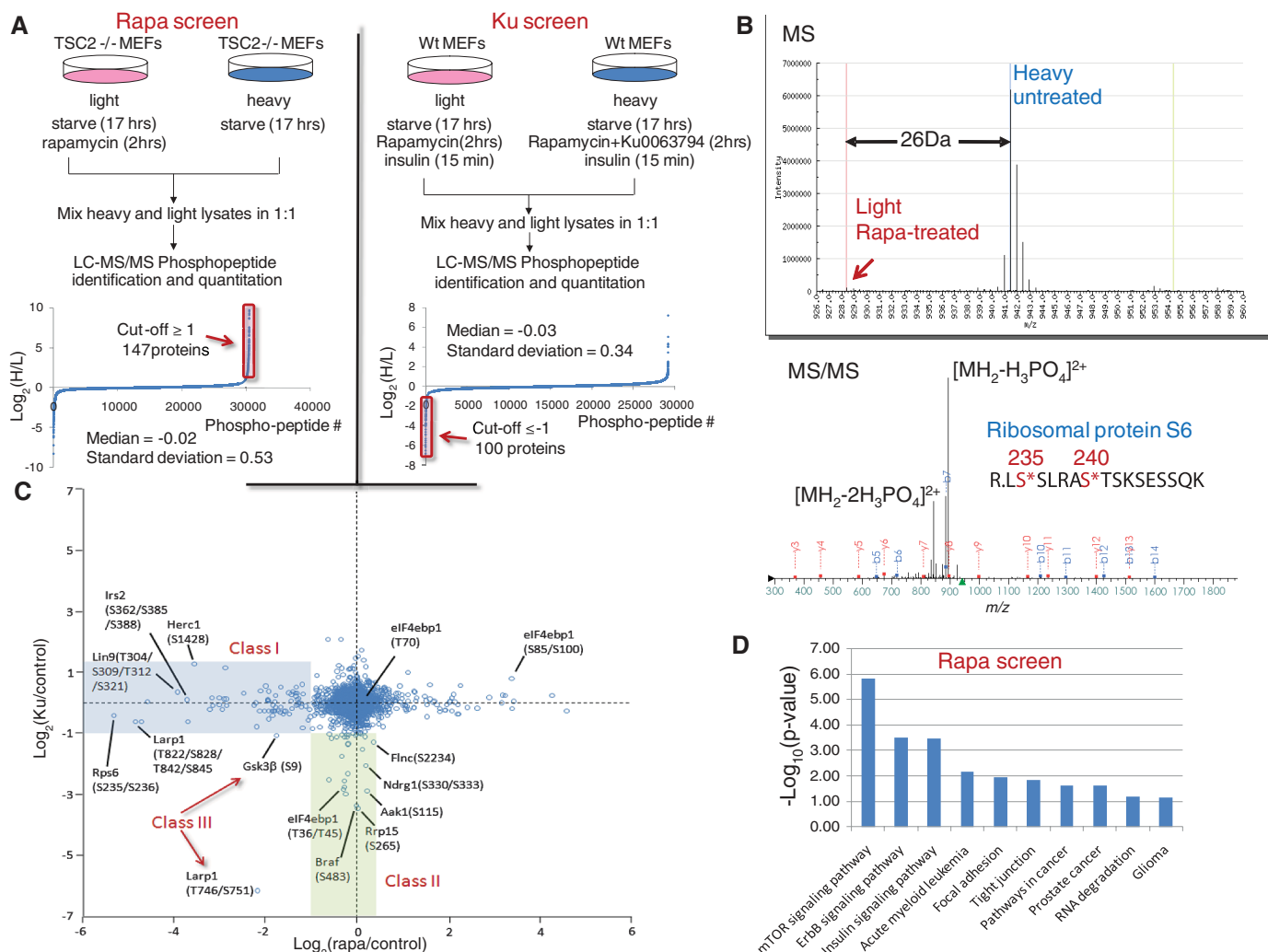
Several hundred peptides corresponding to 85 and 147 proteins in the two replicates (database S3 and fig. S1) were determined to contain rapamycin-sensitive phosphorylation sites (defined as phosphorylated peptides in control cells whose abundance were more than twice that in samples from rapamycin-treated cells). Many known effectors of the canonical mTORC1 signaling pathway were identified in the down-regulated population, including p70S6K, 4EBP1/2, Akt1s1 (PRAS40), rpS6, eIF4B, eIF4G1, and GSK3 $\beta$  (table S2 and Fig. 1, C and D). A representative identification of the known rapamycin-sensitive phosphorylation sites on rpS6 is shown in Fig. 1B. In addition, the identification of many kinases, for example, unc-51-like kinase 1 (ULK1),

in the down-regulated proteins provides potential points for signal integration and crosstalk (table S2) [see SOM text and table S3 for Gene Ontology (GO) analysis and detailed discussion of the hits].

Rapamycin is an allosteric inhibitor that only partially inhibits mTORC1 signaling and has no effect on the activity of mTORC2 under short-term treatment conditions (2). In contrast, adenosine triphosphate (ATP)-competitive mTOR inhibitors block the activity of both mTORC1 and mTORC2 (1). To identify rapamycin-insensitive mTORC1, and mTORC2 substrates, we used the mTOR inhibitor Ku-0063794 and performed a second SILAC experiment (Ku screen) (Fig. 1, A and C, and fig. S1). In this experiment, 100 pro-

teins were determined to contain down-regulated phosphopeptides after Ku-0063794 treatment (database S3 and table S2) (see SOM text for detailed discussion).

One of the enriched GO classes of hits in the Rapa screen is the receptor protein tyrosine kinase (RTK) signaling pathway ( $P = 0.01$ , table S3), suggesting that mTORC1 might modulate its upstream regulators by altering the activities of RTKs. In particular, phosphorylation of S501 and S503 on the growth factor receptor-bound protein 10 (Grb10) was strongly inhibited by a 2-hour rapamycin treatment (Fig. 2A, fig. S4A, and table S2). The intensity of a triply phosphorylated Grb10 peptide (T76, S96, and S104) also decreased after rapamycin treatment (table S2).



**Fig. 1.** Sample preparation and data analysis for quantitative phosphoproteomic profiling of the mTOR downstream signaling networks. **(A)** Schematic of the two SILAC mass spectrometry experiments are shown with a plot highlighting the ratio distribution of phosphopeptides identified in each screen (see data summary in table S1). Note that most of the phosphopeptides have a ratio of 1:1 between the light and heavy populations and hence have a value close to 0 on a  $\log_2$  axis. Proteins with down-regulated phosphorylation in each screen are highlighted in the red box. **(B)** Typical mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra in which LS\*SLRAS\*TSKSESSQK from ribosomal protein S6 (S235 and S240)

was identified as a rapamycin-sensitive phosphopeptide. The light and heavy peptides differ by 26 daltons, corresponding to two labeled Lys and one labeled Arg in this particular peptide. **(C)** Quantitative differences between the rapamycin-sensitive and insensitive mTOR downstream phosphorylation events. Phosphopeptides identified in both screens were extracted and their corresponding treatment/control ratios (see table S1 for treatment conditions) were plotted on a  $\log_2$  scale.  $\log_2(\text{treatment/control}) \leq -1$  is considered to be down-regulated (see SOM text for detailed discussion). **(D)** The top 10 pathways enriched in the down-regulated phospho-proteins identified in the Rapa screen.

We developed a phospho-specific antibody (fig. S5, A and B) and found that rapamycin treatment induced rapid dephosphorylation of Grb10 at S501 and S503 (Fig. 2B). Grb10 phosphorylation was also decreased in *Tsc2*<sup>-/-</sup> MEFs deprived of amino acids (Fig. 2C). To determine whether S501 and S503 of Grb10 can be phosphorylated by other kinases, we treated *Tsc2*<sup>-/-</sup> cells with staurosporine, a broad-spectrum kinase inhibitor that, however, does not suppress mTOR activity (7). No change in the phosphorylation of Grb10 was observed (Fig. 2D). S6K activity was inhibited by staurosporine treatment, as shown by a complete loss of rpS6 phosphorylation, suggesting that Grb10 was directly phosphorylated by mTORC1 rather than by S6K.

In wild-type (WT) MEFs, insulin or serum stimulation increased Grb10 phosphorylation in a rapamycin-sensitive manner (Fig. 2E). Akt inhibition also reduced Grb10 and S6 phosphorylation. Grb10 S503 can be phosphorylated by ERK in vitro (8). Inhibition of the ERK-activating kinase MEK with AZD6244 abolished phosphorylation of ERK but had no effect on phosphorylation of Grb10 (Fig. 2E), indicating that phosphorylation of Grb10 (Fig. 2E), indicating that phosphorylation

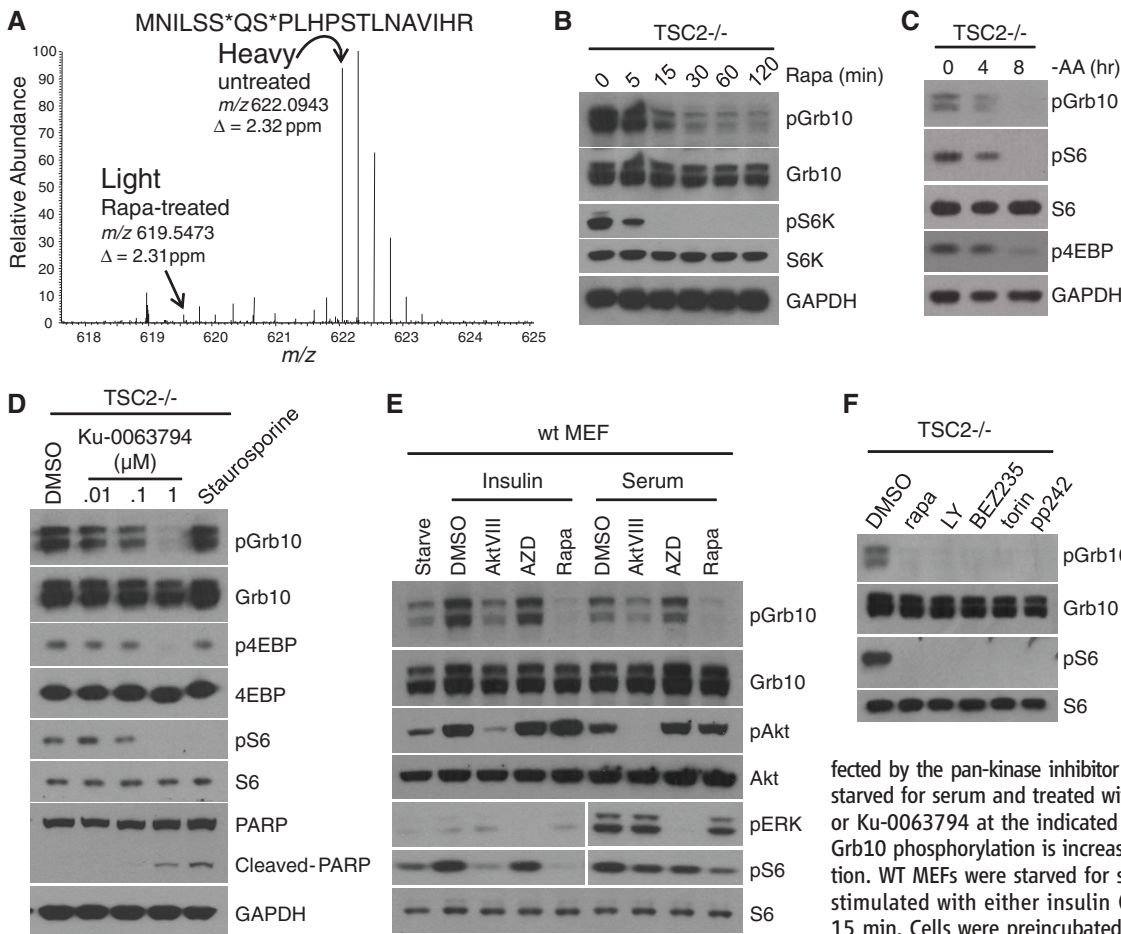
of S501 and S503 on Grb10 is not mediated by ERK in vivo. All other mTOR catalytic inhibitors tested, including LY294002, NVP-BEZ235, torin, and pp242 (9), also completely abolished Grb10 phosphorylation (Fig. 2F).

To examine a potential interaction between Grb10 and the mTOR complexes in vivo, we expressed hemagglutinin (HA)-tagged Grb10 with Myc-tagged raptor or rictor in human embryonic kidney (HEK) 293T cells. Grb10 interacted with raptor, but not rictor, indicating that Grb10 is a binding partner of mTORC1, but not mTORC2 (Fig. 3A). Grb10 was also phosphorylated by recombinant mTOR at S501 and S503 in vitro (Fig. 3B).

Grb10 was much more abundant in *Tsc2*<sup>-/-</sup> and *Tsc1*<sup>-/-</sup> MEFs than in their wild-type counterparts (Fig. 3C and fig. S5C), and the initial loss of Grb10 phosphorylation as a result of rapamycin treatment was followed by a decrease in Grb10 abundance and a smaller decrease in the amount of Grb10 mRNA (Fig. 3D). Exposure to a proteasome inhibitor MG-132 suppressed rapamycin-induced Grb10 protein degradation (fig. S5D). These results show that mTORC1 functions to promote accumulation of Grb10 both transcrip-

tionally and posttranslationally. Depletion of the mTORC1 component raptor also led to decreased abundance of Grb10 protein (Fig. 3E). Furthermore, long-term treatment with mTOR catalytic inhibitors led to reduced levels of Grb10 in *Tsc2*<sup>-/-</sup> MEFs (fig. S5E), *Tsc1*<sup>-/-</sup> MEFs (fig. S5F), and HeLa cells (fig. S5G).

To explore whether Grb10 S501 and S503 phosphorylation contributed to its stabilization and high expression, we transfected WT Grb10, Grb10-S501A-S503A (AA), and Grb10-S501D-S503D (DD) into HEK293T cells. Exogenous WT and DD Grb10 proteins were expressed in similar amount, but the AA mutant of Grb10 was less abundant, perhaps due to protein instability (Fig. 3F). To confirm this result, we generated *Tsc2*<sup>-/-</sup> MEFs stably expressing the HA-tagged Grb10-DD. Long-term rapamycin treatment of these cells decreased amounts of the endogenous, WT Grb10 but had no effect on the abundance of the DD mutant protein (Fig. 3G). This result appears not to result from protein overexpression, because in *Tsc2*<sup>-/-</sup> cells expressing HA-tagged WT Grb10, rapamycin treatment decreased the abundance of both the endogenous



**Fig. 2.** Sensitivity of phosphorylation of Grb10 at S501 and S503 to rapamycin inhibition. **(A)** Identification of a doubly phosphorylated, rapamycin-sensitive Grb10 peptide (MNILSS\*QS\*PLHPSTLNAVIHR; asterisk indicates the site of phosphorylation at S501 and S503). **(B)** Phosphorylation of Grb10 at S501 and S503 shows rapamycin sensitivity in vivo. *Tsc2*<sup>-/-</sup> cells were starved for serum and treated with 20 nM rapamycin for the indicated times. **(C)** Phosphorylation of Grb10 at S501 and S503 is sensitive to amino acids withdrawal. *Tsc2*<sup>-/-</sup> cells were serum-deprived in Dulbecco's modified Eagle's medium (DMEM) overnight and then transferred to a media of DMEM minus amino acids for the indicated times. **(D)** Phosphorylation of Grb10 at S501 and S503 is not affected by the pan-kinase inhibitor staurosporine. *Tsc2*<sup>-/-</sup> cells were starved for serum and treated with either 100 nM staurosporine or Ku-0063794 at the indicated concentrations for 2 hours. **(E)** Grb10 phosphorylation is increased upon growth factor stimulation. WT MEFs were starved for serum overnight and then were stimulated with either insulin (100 nM) or serum (10%) for 15 min. Cells were preincubated with the indicated compounds for 2 hours. AktVIII (1 μM) and AZD (AZD6244, 5 μM) are specific

inhibitors of Akt and MEK, respectively. Rapa (rapamycin) was used at 20 nM. **(F)** Grb10 phosphorylation at S501 and S503 is sensitive to various mTOR kinase inhibitors. *Tsc2*<sup>-/-</sup> cells were serum-starved and treated with the indicated compounds for 2 hours. The concentrations of the compounds were Rapa (rapamycin), 20 nM; LY (LY294002), 20 μM; BEZ235 (NVP-BEZ235), 500 nM; torin, 100 nM; and pp242, 1 μM. Phosphorylation levels in this figure were measured with phospho-specific antibodies against Grb10 (S501 and S503), S6K (T389), S6 (S235 and S236), Akt (S473), 4EBP (T37 and T46) and ERK1/2 (T202 and Y204).



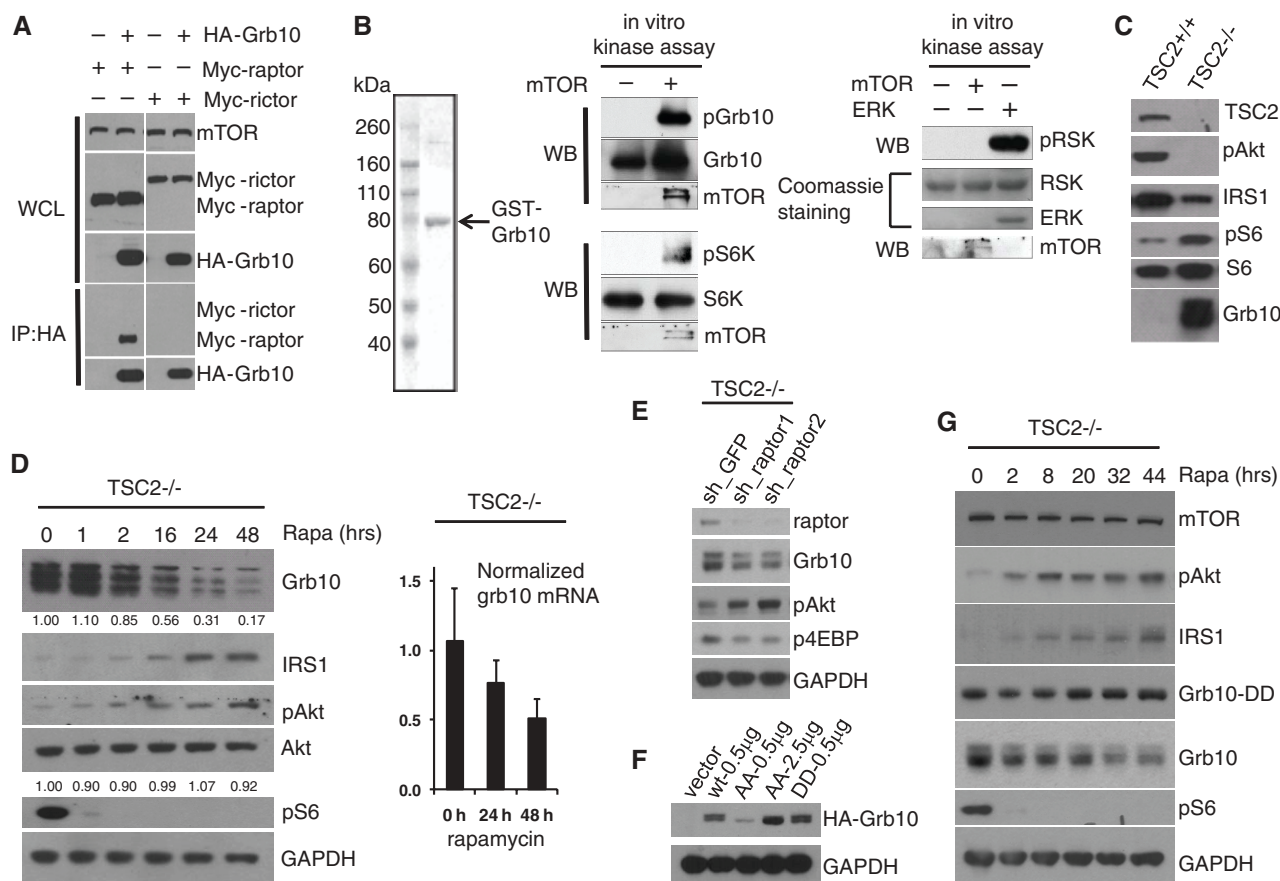
and ectopically expressed Grb10 (fig. S5H). These data support a critical role for mTORC1 in stabilizing Grb10 through phosphorylation of the S501 and S503 residues. It is important to note that phospho-Akt levels still increased in these cells (Fig. 3G), likely resulting from increased insulin receptor substrate 1 (IRS1) levels after prolonged rapamycin treatment.

Grb10 functions as a negative regulator of insulin signaling. In Grb10 null mice, PI3K-Akt pathway hyperactivation was observed in insulin-sensitive tissues (10, 11). We therefore examined the possibility that mTORC1-mediated Grb10 phosphorylation and accumulation activated a negative feedback loop from mTORC1 to the PI3K-Akt pathway. The PI3K-Akt and ERK-MAPK pathways were both refractory to insulin or insulin-

like growth factor (IGF) stimulation in *Tsc2*<sup>-/-</sup> cells, as a result of constitutively elevated mTORC1 signaling (5, 6). In contrast, phosphorylation of both Akt and ERK was increased in Grb10-depleted cells deprived of serum or stimulated with insulin or IGF (Fig. 4A and fig. S6A). Conversely, overexpression of Grb10 in HEK293 cells suppressed activation of PI3K (fig. S6B) by inhibiting insulin receptor-dependent phosphorylation of IRS and its subsequent recruitment of PI3K (fig. S6, C and D) (12). Knockdown of Grb10 in *Tsc2*<sup>-/-</sup> MEFs to a level close to that in WT cells did not completely restore the sensitivity of PI3K to insulin stimulation (fig. S6E), suggesting that additional mechanisms (e.g., lower IRS levels in *Tsc2*<sup>-/-</sup> MEFs) contribute to the feedback inhibition (fig. S6F). Our data complement the previous findings

and suggest that activation of mTORC1-S6K promotes negative feedback inhibition of PI3K through a two-pronged mechanism: first, mTORC1-S6K-mediated phosphorylation and degradation of a positive regulator of PI3K signaling, IRS (6, 13, 14); second, mTORC1-mediated phosphorylation and accumulation of a negative regulator of PI3K signaling, Grb10.

We next asked whether PI3K activation in Grb10-depleted cells would promote survival against stress-induced apoptosis. In response to either staurosporine or etoposide, reduced caspase 3 cleavage was observed in Grb10 knockdown cells compared with that of control cells, indicating that Grb10 depletion is sufficient to protect cells from apoptosis (Fig. 4B and fig. S6G). Because rapamycin can protect cells from energy

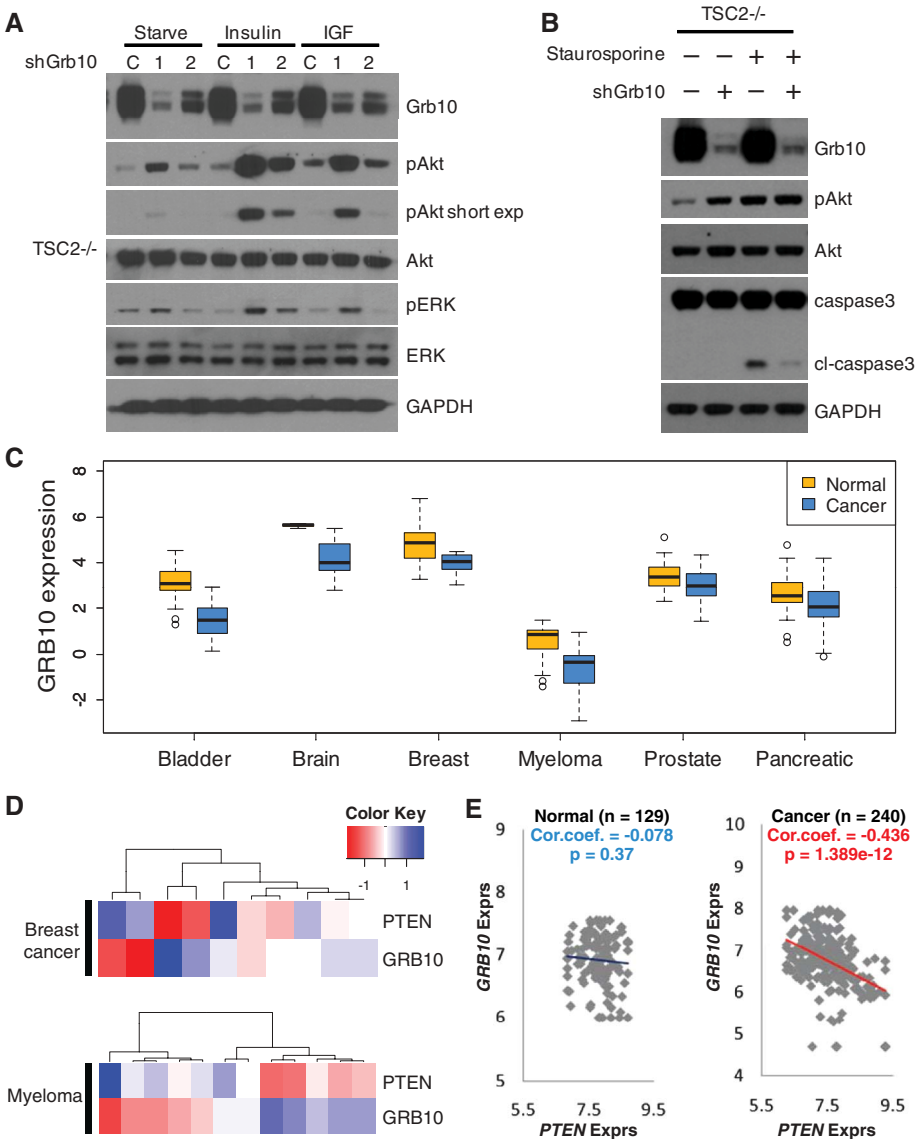


**Fig. 3.** Effect of mTOR-mediated phosphorylation to promote stability of Grb10. (A) Grb10 interacts with raptor, but not rictor. HA-tagged Grb10 was transfected with Myc-raptor or Myc-rictor into HEK293T cells. Cells were lysed in lysis buffer A, and the lysates were subjected to immunoprecipitation using antibody to HA conjugated beads. Raptor and rictor were probed with an antibody against the Myc-tag. WCL, whole cell lysates. (B) Grb10 is phosphorylated by mTOR in vitro. Recombinant GST-Grb10 was prepared from bacteria and was incubated with recombinant mTOR in vitro. Phosphorylation of Grb10 at S501 and S503 was detected by using the phospho-specific antibody against these two sites. S6K and ribosomal protein S6 kinase (RSK) were used as the positive and negative controls, respectively, and the experiments were performed in parallel with the mTOR-Grb10 in vitro kinase assay. WB, Western blotting. (C) Grb10 is highly overexpressed in *Tsc2*<sup>-/-</sup> cells. (D) Long-term rapamycin treatment leads to Grb10 degradation in *Tsc2*<sup>-/-</sup> cells. Grb10 protein

expression levels inversely correlated with Akt activity. mRNA level was determined using quantitative reverse transcription polymerase chain reaction based on three biological replicate experiments. (E) Knockdown of raptor in *Tsc2*<sup>-/-</sup> cells decreased Grb10 protein level. Cells were starved overnight, and the lysates were probed with the antibodies indicated. (F) S501A-S503A mutant is unstable compared with the WT or the S501D-S503D mutant. The indicated amount of DNA was transfected into HEK293T cells. (G) Rapamycin failed to induce degradation of the S501D-S503D mutant. S501D-S503D mutant (DD) was stably expressed in *Tsc2*<sup>-/-</sup> cells, and cells were treated with 20 nM rapamycin for the indicated times. Endogenous Grb10 was detected using an antibody that preferentially recognizes mouse Grb10, whereas the Grb10 DD mutant (of human origin) was detected using an antibody to HA. Phosphorylation levels in this figure were measured with phospho-specific antibodies against Grb10 (S501 and S503), S6K (T389), RSK (T573), Akt (S473), and 4EBP (T37 and T46).

stress-induced death (15), these results provide additional possible explanations for the cytostatic rather than cytotoxic effects of rapamycin in some cancers and suggest that a complete understanding of the feedback inhibition control will be critical in designing combination therapies involving rapamycin analogs.

Comprehensive meta-analysis of published microarray data revealed that the abundance of *GRB10* was decreased in many tumor types compared with that in normal tissue counterparts (Fig. 4C). Given that loss of Grb10 results in activation of the PI3K-Akt pathway (Fig. 4A), we performed correlation analysis and found that there was a significant ( $P < 0.05$ ) negative correlation between *GRB10* and *PTEN* expression (Fig. 4, D and E). This correlation was only observed in tumor samples but not in normal tissue controls (Fig. 4E). *PIK3CA* mutations and *PTEN* loss are mutually exclusive in breast cancer (16), suggesting that increased abundance of phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) resulting from of genetic alteration of either *PIK3CA* or *PTEN* relieves selective pressure targeting the other gene. Similarly, loss of Grb10, which results in PI3K activation, might provide the cells with growth and survival advantages that are redundant with respect to *PTEN* loss-of-function, suggesting that Grb10 might be a tumor suppressor that is regulated by mTORC1. These data point to the prospect of targeting Grb10 stability in cancer therapy.



**Fig. 4.** Grb10 is involved in the feedback inhibition loop from mTORC1 to PI3K and ERK-MAPK, and *GRB10* mRNA expression is decreased in abundance in many cancers and is negatively correlated with *PTEN* expression. (A) Knockdown of Grb10 in *Tsc2*<sup>-/-</sup> cells resulted in PI3K and ERK-MAPK hyperactivation after insulin or IGF stimulation. C, shGFP; 1, shGrb10 #1; 2, shGrb10 #2. Phosphorylation levels were measured against Akt (S473) and ERK1/2 (T202 and Y204). (B) Knockdown of Grb10 in *Tsc2*<sup>-/-</sup> cells protected cells against apoptosis. Grb10 knockdown and control cells were starved overnight and then treated with 100 nM staurosporine for 5 hours to induce apoptosis. Phosphorylation levels were measured against Akt (S473). (C) Box plots indicating that *GRB10* expression is significantly lower in many tumor types compared with their corresponding normal tissues. (Only the tumor types that showed significantly lower *GRB10* expression in cancer versus normal in at least three independent microarray data sets are included;  $P < 0.01$ , Log-rank test.) (D) Heat maps indicating a strong negative correlation between *GRB10* and *PTEN* expression in myelomas and breast carcinomas. Low levels of *GRB10* expression rarely occurred in tumors that also showed low levels of *PTEN* expression. The z scores (from -1 to +1) of the normalized expression values for the corresponding cancer data sets in (C) are shown. Red, lower expression compared to mean (white). Blue, higher expression compared to mean (white). (E) Scatter plots comparing the expression levels of *GRB10* and *PTEN* in the normal and tumor samples, collected from six different tissue types indicated in (C). The negative correlation between *GRB10* and *PTEN* expression is evident in the tumor but not in the corresponding normal samples.

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Supporting Online Material

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# Adaptation and Evolutionary Rescue in Metapopulations Experiencing Environmental Deterioration

Graham Bell and Andrew Gonzalez\*

It is not known whether evolution will usually be rapid enough to allow a species to adapt and persist in a deteriorating environment. We tracked the eco-evolutionary dynamics of metapopulations with a laboratory model system of yeast exposed to salt stress. Metapopulations experienced environmental deterioration at three different rates and their component populations were either unconnected or connected by local dispersal or by global dispersal. We found that adaptation was favored by gradual deterioration and local dispersal. After further abrupt deterioration, the frequency of evolutionary rescue depended on both the prior rate of deterioration and the rate of dispersal. Adaptation was surprisingly frequent and rapid in small peripheral populations. Thus, evolutionary dynamics affect both the persistence and the range of a species after environmental deterioration.

Global environmental change is causing elevated rates of population extirpation (1, 2), giving rise to concern that the rate of environmental change may exceed the capacity of populations to persist and maintain their range (3). A population that is exposed to a stress may adapt through natural selection and thereby avoid extinction (4–7). This process of evolutionary rescue has been documented for single populations in the laboratory (8) but has not been studied in metapopulations representing the range of a species. Hence, it is not known whether evolutionary rescue can occur across metapopulations to prevent range collapse and species extinction. Patterns of range collapse (9, 10) show that species can persist in the periphery of their historical range. Peripheral populations tend to show lower levels of genetic diversity than central populations, although this difference is not large (11). Theory indicates how species will respond to stress, as their range collapses or expands or shifts in space (12), but these predictions remain untested experimentally. Here we describe an experiment showing that adaptation to environmental deterioration is most likely to evolve when change is slow and individuals can disperse. Consequently, the history and spatial structure of metapopulations affect the likelihood of evolutionary rescue and thereby the maintenance of the species range.

We set up model ranges of baker's yeast on standard 96-well plates where each well constituted a population (13). Yeast is increasingly being developed as a model organism in ecology and evolution (8, 14–16). We maintained the populations in continual growth by transferring a small quantity from each well at regular intervals to the corresponding well on a new plate supplied with fresh growth medium. We stressed the pop-

ulations by adding different amounts of salt to the growth medium to create a gradient across the plate, with more favorable conditions (low salt concentration) to the “south” and more stressful conditions (high salt concentration) to the “north” (fig. S1). This created a corresponding gradient of population density, higher in the south than in the north. We then intensified this stress by shifting the gradient to the south between transfers, compressing the range and eventually creating conditions in the far north of the plate that would be lethal to the ancestral populations. This enabled us to track shifts in population size and study evolutionary rescue across the metapopulation.

The likelihood of evolutionary rescue in a metapopulation will depend on the quantity of genetic variation, the rate of environmental deterioration, and the rate of dispersal between individual populations. Our yeast populations were derived from a single colony and grew asexually, so that genetic variation was at first minimal and was thereafter generated by mutation and changes in gene expression (17). Almost 500 loci contribute to tolerance of high concentrations of salt in yeast (18), and about 60 have severely reduced competitive ability in saline medium (19), so the loci that potentially contribute to evolutionary rescue at high salt concentration make up at least 1% of the yeast genome. Candidates for genes involved in evolutionary rescue at high salt concentration were previously identified by functional profiling (8, 19). We imposed three rates of environmental deterioration: zero (“constant” treatment, gradient unchanged between transfers), low (“slow” treatment, gradient shifted southward every four transfers), and high (“fast” treatment, shifted every two transfers). In each case, there were three modes of dispersal from well to well during each transfer: no dispersal (each well is inoculated exclusively from the corresponding well on the old plate), local dispersal (each well receives a small contribution from wells with the next lower salt concentration on the old plate), and global dispersal (each well receives a small

contribution from all the wells on the old plate). Hence, the experiment comprised nine combinations of rate of deterioration and mode of dispersal. It was conducted in two phases. In the first phase, we followed the dynamics of adaptation by recording the yield (cell density at the end of the growth cycle) (13) of each population as the environment deteriorated at the prescribed rate. In the second phase, we transferred each experimental plate regardless of its history to the same severe gradient to measure the frequency of evolutionary rescue across all the populations in the range.

Yield at first declined linearly from south to north across the plate in response to increasing salt concentration. The rate of decline and the spatial distribution of yield changed over time as the populations evolved (Fig. 1). There was no statistical interaction between the rate of environmental deterioration and the mode of dispersal [analysis of variance (ANOVA),  $F_{4,4} = 1.69$ ,  $P = 0.18$ ]. The rate of change in mean yield over the whole range varied with the rate of environmental deterioration (ANOVA,  $F_{2,2} = 625.45$ ,  $P < 0.001$ ). It increased slightly in the constant treatment, and declined substantially under slow deterioration and much more rapidly under rapid deterioration. This was modulated by dispersal (ANOVA,  $F_{2,2} = 8.74$ ,  $P = 0.0012$ ) because local dispersal extended the habitable zone northward and slowed the decline of yield relative to both no dispersal and global dispersal (fig. S2).

Within the metapopulation there is a pronounced U-shaped relationship between the rate of change in yield and the level of stress, such that populations exposed to intermediate salt concentration declined more rapidly than those at very low or very high concentration. This was apparent in the constant treatment (fig. S3) and was exaggerated by environmental deterioration (Fig. 2 and table S1). The effect is modest at low concentration, amounting to a few percent of overall yield, but is much more substantial, amounting to 30% or more of overall yield, at high concentration. This U-shaped relationship arises because the rate of adaptation to stress is necessarily constrained by the concomitant reduction in population size. Smaller populations are expected to have a lower overall mutation supply rate and therefore to adapt more slowly to a stressor such as salt (20). The fraction of these mutations that are beneficial with respect to a specific stress depends on the state of adaptedness of the population. In benign conditions to which a population is well adapted, there may be no mutations available that would further increase growth. In mildly stressful conditions a few beneficial mutations may exist, and the fraction of mutations that are beneficial will increase with the level of stress, up to the point where the breakdown of fundamental biological processes hinders or entirely prevents any alleviation. The overall rate of beneficial mutation is the product of the mutation supply rate, which falls with increasing stress, and the fraction of mutations that are beneficial, which rises with increasing stress up

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to some limit. Consequently, the rate of adaptation may be a U-shaped function of stress, with populations subjected to mild stress adapting slowly, or failing to adapt at all, whereas those subjected to severe stress adapt more rapidly (fig. S4). The effect of environmental deterioration is to expose a new range of beneficial mutations in populations that had become adapted to worsening conditions (21, 22). This shifts the curve relating the fraction of beneficial mutations to the level of stress to the right, thus deepening the U-shaped pattern of adaptation (Fig. 2 and fig. S5). This was so pronounced that we observed a positive response to selection only under nearly lethal levels of stress at the edge of the range, a surprising effect that was observed even in isolated populations experiencing rapid environmental deterioration.

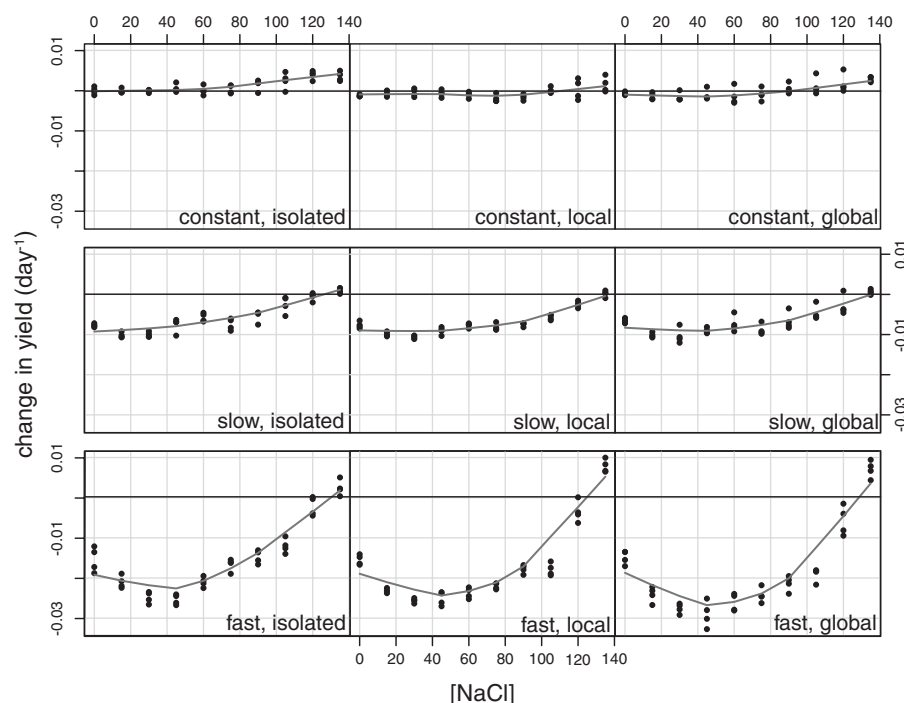
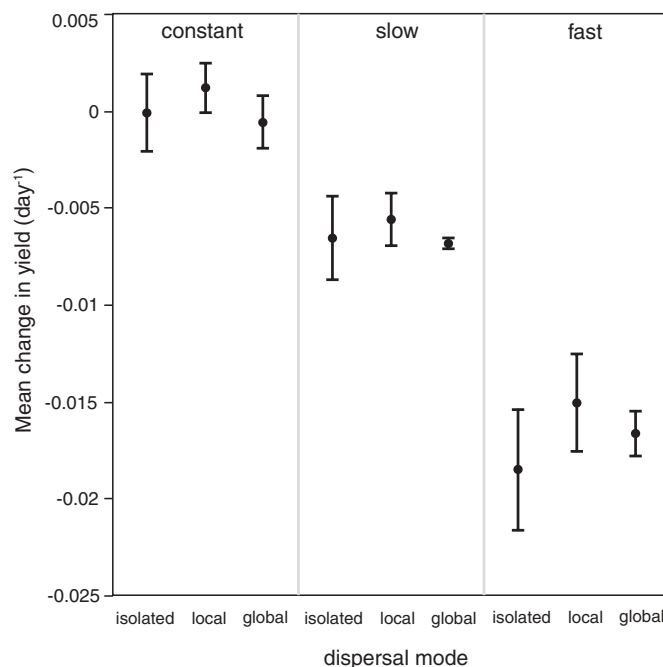
In the second phase of the experiment a further episode of deterioration, of the same magnitude as those of previous episodes, was imposed on all the experimental ranges and resulted in lethal conditions appearing in the southern section of the plates (13). For the slow and fast treatments, this was a continuation of the previous tempo of deterioration, whereas for the constant treatment it represented an abrupt severe stress. Subsequently, we often observed the abrupt decline in yield followed by an increase typical of population recovery by evolutionary rescue. We expected that the frequency of rescue would be increased by prior selection in a deteriorating environment because beneficial mutations conferring tolerance to lethal conditions would have spread by virtue of their correlated advantage at sublethal levels of stress. We further expected that rescue would be facilitated by dispersal, because beneficial mutations that had spread in other populations could be introduced into poorly adapted populations as immigrants.

The rate of environmental deterioration and mode of dispersal interact to affect the frequency of rescue (Fig. 3 and table S2). We scored a rescue event when the yield of a population after three transfers exceeded its yield just before the abrupt stress. The fewest events occurred in unconnected ranges that had previously experienced no environmental change [mean  $\pm$  95% confidence interval (CI) for constant, zero-dispersal treatments:  $25.25 \pm 4.9$ ]. Both local and global dispersal significantly increased the mean number of rescue events in the constant environment (local dispersal:  $35.75 \pm 5.85$ ; global dispersal:  $49.75 \pm 6.91$ ). Environmental deterioration before abrupt environmental change markedly increased the mean number of evolutionary rescue events. This was most apparent in unconnected metapopulations (no dispersal: constant =  $25.25 \pm 4.9$ , slow =  $40.75 \pm 6.25$ , and fast =  $45.75 \pm 6.62$ ); dispersal did not significantly improve the number of rescue events in the presence of historical environmental change. Hence, the likelihood of evolutionary rescue depends on both the history of environmental change and the connectivity of the metapopulations.

A history of environmental deterioration also affected the degree of adaptation that evolved after abrupt change (Fig. 4 and table S3). In the constant treatment, there was a U-shaped relationship between population growth rate and salt

concentration, as in the first phase of the experiment, indicating evolutionary rescue in populations at the extreme ends of the stress gradient. In metapopulations that had previously experienced environmental deterioration, whether slow or fast,

**Fig. 1.** The effect of environmental change and dispersal on average ( $\pm 95\%$  CI) rate of change in yield across all 60 populations per metapopulation over 12 transfers.



**Fig. 2.** The mean rate of change in yield across the gradient of stress (NaCl, g/liter). Salt concentration is the initial value for the treatment: in deteriorating environments, this concentration rises over time. The panels show the patterns of response in relation to treatment combinations [(13), and main text for treatment descriptions] of environmental change (top to bottom: constant, slow, and fast) and dispersal mode (left to right: no dispersal, local, and global). The gray lines show the locally weighted polynomial regression (lowess) fit, and the horizontal black lines provide a guide for no change in yield. The panel at top right is reproduced in more detail as fig. S3.

the mean rate of change in yield was positive at all points along the concentration gradient, showing the overwhelming effect of prior adaptation to severe stress in enhancing adaptation at lethal levels of stress. The curvilinear relation between adaptation and stress is no longer apparent; instead, adaptation increases steadily from lower to higher salt concentrations. This is expected because adaptation increases population size, making

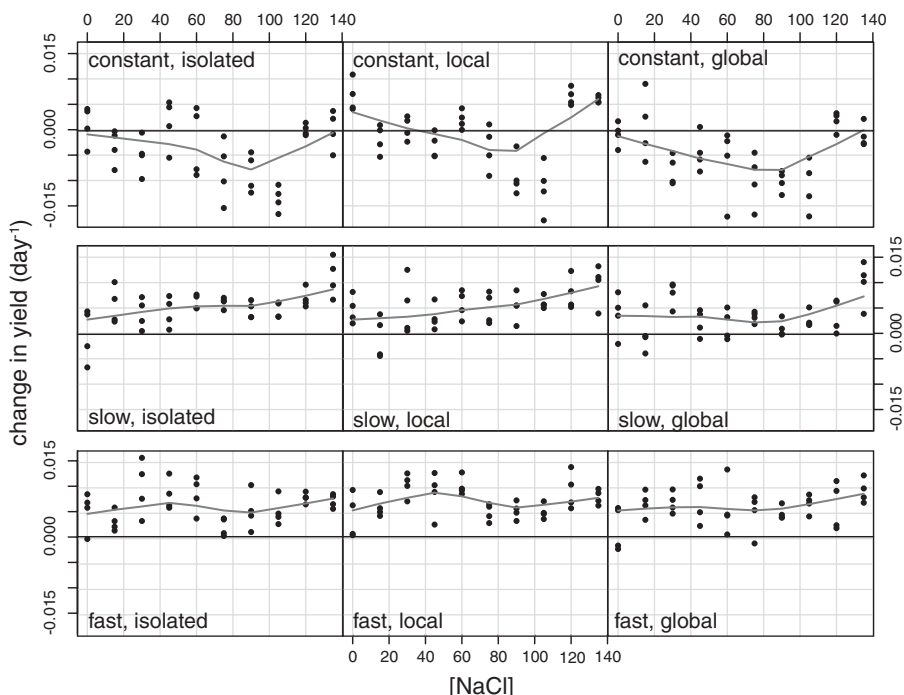
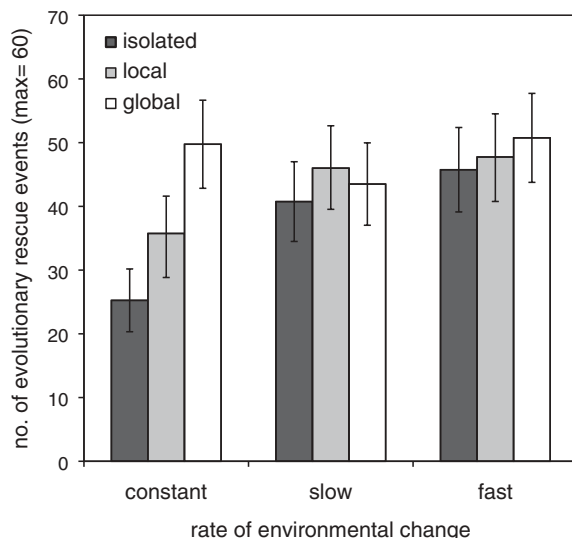
the relation between stress and the mutation supply rate more shallow, and thereby tending to establish a monotonic relation between stress and adaptation (fig. S6).

Here we have shown that slow prior deterioration and modest levels of dispersal can foster the ability of a species to evolve tolerance to environmental stress sufficient to extirpate its ancestor. The mechanism responsible appears to

be the spread of beneficial mutations conferring adaptation to intermediate levels of stress, which have the correlated effect of also conferring some degree of tolerance to severe stress before this is actually experienced, as previously documented for the yeast-salt system in single populations (15). Local dispersal transfers these alleles up the stress gradient and thereby prevents the collapse of the metapopulation; global dispersal is sometimes less effective because both the mean fitness of immigrants and the probability of introducing a well-adapted mutant are much lower. Populations embedded within connected metapopulations that have experienced historical environmental change have a greater probability of recovering and persisting by evolutionary rescue after a severe perturbation.

Our microcosms are incomplete models of metapopulations occupying natural landscapes, where other processes such as interactions between species or between combinations of stressors also influence range dynamics (23). They are an important first step toward developing more realistic laboratory models using automated transfer systems with high levels of replication to analyze the factors responsible for adaptation and evolutionary rescue in deteriorating environments (24). We believe that such experiments can provide the basis for the synthesis of spatial ecology with evolution that is required to understand the impacts of anthropogenic stress on the Earth's biodiversity.

**Fig. 3.** The effect of dispersal (isolated, local, and global) and historical environmental change (constant, slow, and fast) on the mean ( $\pm 95\%$  CI) number of evolutionary rescue events (maximum = 60) across the metapopulations after abrupt environmental change.



**Fig. 4.** The effect of past environmental change and dispersal mode on the mean rate of change in yield over four transfers after abrupt environmental change. The panels show the patterns of response to salt (NaCl, g/liter) stress in relation to treatment combinations [(13), and main text for treatment descriptions] of environmental change (rows top to bottom: constant, slow, and fast) and dispersal mode (columns left to right: no dispersal, local, and global). The gray lines show the locally weighted polynomial regression (lowess) fit, and the horizontal black lines provide a guide for no change in yield.

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/332/6035/1327/DC1  
Materials and Methods

Figs. S1 to S6

Tables S1 to S3

Additional Data tables S1 to S3  
References (25–29)

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# Nicotine Decreases Food Intake Through Activation of POMC Neurons

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Smoking decreases appetite, and smokers often report that they smoke to control their weight. Understanding the neurobiological mechanisms underlying the anorexic effects of smoking would facilitate the development of novel treatments to help with smoking cessation and to prevent or treat obesity. By using a combination of pharmacological, molecular genetic, electrophysiological, and feeding studies, we found that activation of hypothalamic  $\alpha 3\beta 4$  nicotinic acetylcholine receptors leads to activation of pro-opiomelanocortin (POMC) neurons. POMC neurons and subsequent activation of melanocortin 4 receptors were critical for nicotine-induced decreases in food intake in mice. This study demonstrates that nicotine decreases food intake and body weight by influencing the hypothalamic melanocortin system and identifies critical molecular and synaptic mechanisms involved in nicotine-induced decreases in appetite.

Smoking remains the leading cause of preventable death in developed countries (1), and some smokers report that they smoke as a method of weight control (2, 3). Smokers have a notably lower body mass index than nonsmokers (4) and gain weight when they quit (5). These effects on body weight have been attributed to the nicotine in tobacco, because nicotine decreases feeding in animal models (6). Nicotine has some effects on peripheral energy metabolism (7–9),

but little is known about potential central nervous system pathways mediating nicotine's effects on food intake and body weight. Identifying these pathways could help to study potential cholinergic modulation of appetite and weight control and could also lead to the development of novel appetite suppressants that might aid in smoking cessation.

In a first step toward this goal, we determined that nicotine and the more selective drug cytosine

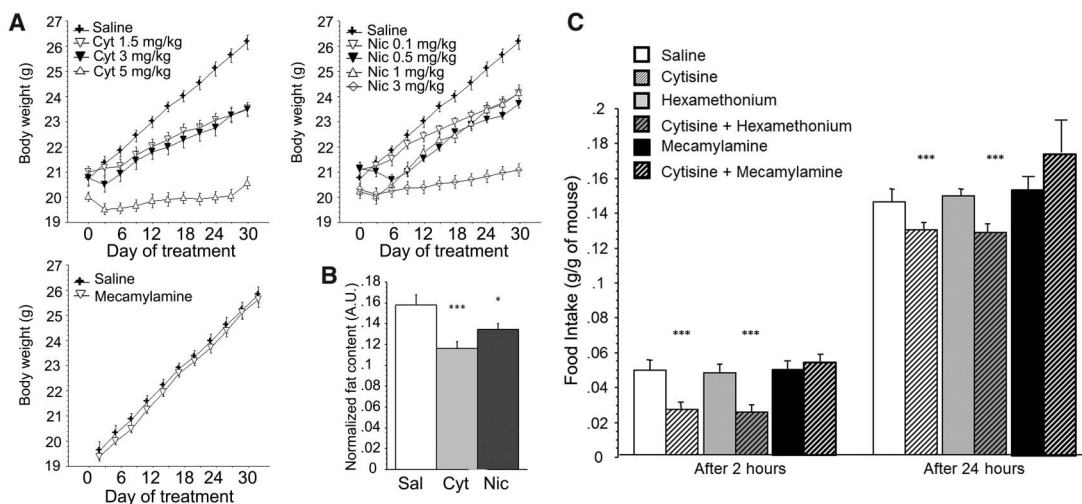
[a full agonist at  $\alpha 3\beta 4$  nicotinic acetylcholine receptors (nAChRs), with weaker effects at other nAChRs (10)], could decrease weight gain over time [Fig. 1A; repeated-measures analysis of variance (ANOVA)  $F(72, 720) = 41.5$ ,  $P < 0.0001$ ], body fat mass by about 15 to 20% [Fig. 1B; ANOVA  $F_{2,26} = 7.7$ ,  $P < 0.001$ ], and food intake by up to 50% [Fig. 1C;  $F_{1,18} = 100.3$ ,  $P < 0.001$ ] in mice but did not affect water intake or tissue water content (fig. S1) (11). In addition, mecamylamine (a noncompetitive nicotinic antagonist) had no effects on its own but prevented acute and chronic cytosine-induced hypophagia (all  $F$  values  $< 1$ ), whereas the non-brain-permeant nicotinic antagonist hexamethonium was ineffective in blocking these anorexic effects (Fig. 1C), suggesting that activation of central nAChRs was essential for reduced food intake.

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**Fig. 1.** Change in weight, body fat content, and food intake after treatment with nicotinic drugs. Both nicotine (Nic) and cytosine (Cyt) dose-dependently limited weight gain in mice treated daily for 30 days, with more pronounced effects seen at higher doses (A) (all  $P$  values  $< 0.001$ ). In contrast, the nonselective nicotinic antagonist mecamylamine had no significant effect ( $F < 1$ ), suggesting that nAChR antagonism alone was not sufficient for the anorexic effects of nicotinic compounds. Error bars indicate SEM. Body fat measured by magnetic resonance imaging was also reduced in mice treated with cytosine [1.5 mg/kg;  $F_{1,23} = 13.7$ ,  $P = 0.006$ ] and nicotine [0.5 mg/kg;  $F_{1,23} = 5.08$ ,  $P = 0.03$ ] compared with saline (Sal) (B). Acute injection of cytosine decreased food intake after 2 hours [ $F_{1,18} = 100.3$ ,  $P < 0.001$ ], and this effect was still observed after 24 hours [ $F_{1,18} = 35.3$ ,  $P < 0.001$ ]. A.U., arbitrary units. The



effect of cytosine was not blocked by the peripherally acting nAChR antagonist hexamethonium [2 hours,  $F_{1,18} = 121.5$ ,  $P < 0.001$ ; 24 hours,  $F_{1,18} = 37.9$ ,  $P < 0.001$ ] but was blocked by mecamylamine ( $F$  values  $< 1$ ) (C), indicating cytosine acts at central nAChRs to exert its anorexic effects. \*\*\* indicates  $P < 0.001$ .

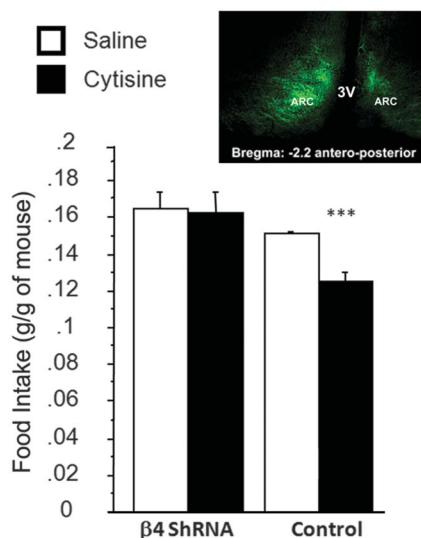


The pharmacological specificity of cytosine and the relatively low dose (1.5 mg/kg) needed to decrease food intake suggested that activation of central  $\alpha 3\beta 4$  nAChRs is essential for the anorexic effects of nicotinic compounds. We investigated this hypothesis by knocking down the expression of the  $\beta 4$  nAChR subunit with a neuron-specific adeno-associated virus (AAV-2) carrying specific short hairpin RNA molecules (shRNAs). Because the arcuate nucleus (ARC) is one of the most critical brain areas involved in feeding behavior

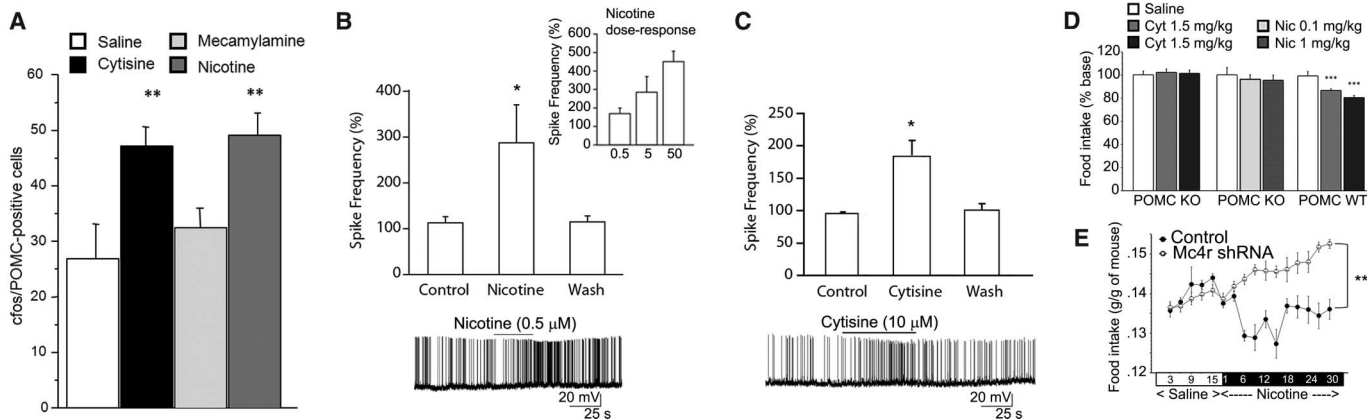
and has been proposed as a potential site for the nicotinic modulation of appetite and food intake (8), we bilaterally infused 0.5  $\mu$ l (1  $\mu$ l per mouse) of high-titer AAV-shRNAs targeting the  $\beta 4$  nAChR subunit into the ventral hypothalamus (Fig. 2) and allowed at least 3 weeks for recovery ( $\beta 4$ KD). We first quantified the efficacy of the knockdown [ $\sim 55 \pm 8.4\%$  (SEM) in mRNA level compared to control (scrambled shRNA); fig. S2A] and did not detect nonspecific effects of the shRNA in other brain regions or at other nAChR subunits (fig. S2, B, C, and D). Mice with  $\beta 4$ KD in ARC were resistant to the effects of cytosine on food intake [ $F_{1,14} = 0.01$ ,  $P = 0.92$ ], whereas  $\beta 2$ KD did not change the anorexic effects of cytosine (Fig. 2 and fig. S3B, respectively).

The ability of  $\beta 4$  nAChR knockdown in the ARC to abolish the anorexic effect of cytosine suggested the involvement of the hypothalamic melanocortin system, an essential brain pathway involved in the regulation of energy balance and food intake (12), as a target for nicotinic drugs. In particular, activation of pro-opiomelanocortin (POMC) cells in the ARC decreases food intake and increases energy expenditure (13), and loss of function of the POMC gene leads to obesity in humans and animals (14, 15). We therefore hypothesized that activation of  $\alpha 3\beta 4$  nAChRs may induce POMC neuron firing, leading to the anorexic effects of nicotinic agonists. We first confirmed that the  $\beta 4$  nAChR is expressed in POMC neurons by using laser-capture microdissection of neurons from transgenic mice expressing green fluorescent protein (GFP) under control of the POMC promoter (fig. S4). We then performed double-labeling immunohistochemical experiments for Fos-like immunoreactivity (FOS-IR) and POMC-IR (fig. S5A) as a measure of neuronal activation in the ARC of mice treated with nicotinic drugs. There was a significant difference across treatments [ $F_{3,12} = 5.85$ ,  $P = 0.0106$ ], and we

found that chronic nicotine and cytosine treatment, unlike mecamylamine, increased FOS-IR selectively in POMC neurons of the ARC by about 50% [Fig. 3A; nicotine  $F_{1,6} = 8.60$ ,  $P = 0.026$ ; cytosine  $F_{1,6} = 7.68$ ,  $P = 0.032$ ] with no detectable effects on other neuronal subtypes in this region (fig. S5B) and that the effect was even greater immediately after an acute injection [about 80%,  $F_{1,14} = 20.73$ ,  $P < 0.001$ ; fig. S5C]. In contrast, there was no significant effect of mecamylamine treatment on FOS- or POMC-IR in the ARC overall [saline versus mecamylamine,  $F_{3,12} = 0.619$ ,  $P = 0.46$ ; for FOS-IR,  $F_{3,12} = 1.39$ ,  $P = 0.29$ ; for POMC-IR  $F_{3,12} = 0.405$ ,  $P = 0.75$ ]. We then identified direct electrophysiological effects of nicotinic drugs on POMC neurons by recording excitatory postsynaptic potentials in the presence of tetrodotoxin (TTX) (0.5  $\mu$ M) and the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor antagonist picrotoxin (50  $\mu$ M) from identified, GFP-labeled POMC neurons in slices from POMC-GFP transgenic mice. Application of nicotine (0.5, 5, 50  $\mu$ M) for 1 to 2 min depolarized the membrane potential and strongly increased the spontaneous firing of POMC neurons to  $289.0 \pm 82.8\%$  of baseline [Fig. 3B;  $F_{2,2} = 4.25$ ,  $P < 0.05$ ]. The nicotine effects were dose-dependent (Fig. 3B, top right). Both 0.5 and 50  $\mu$ M nicotine increased the spike frequency to  $173.4 \pm 27.5\%$  of baseline [ $F_{2,17} = 4.1$ ,  $P < 0.05$ ] and  $456.3 \pm 53.8\%$  of baseline [ $F_{2,17} = 33.65$ ,  $P < 0.001$ ], respectively. Similarly, cytosine (10  $\mu$ M) increased the firing rate of POMC neurons (Fig. 3C), with an average increase of action potential frequency of  $186.2 \pm 23.6\%$  of baseline compared with that of baseline [ $F_{2,29} = 11.3$ ,  $P < 0.01$ ]. Conversely, the nicotinic antagonist mecamylamine did not induce significant changes in the frequency of action potentials in POMC neurons (fig. S6), and, in the presence of TTX (0.5  $\mu$ M) and picrotoxin (50  $\mu$ M), neither nicotine nor cytosine

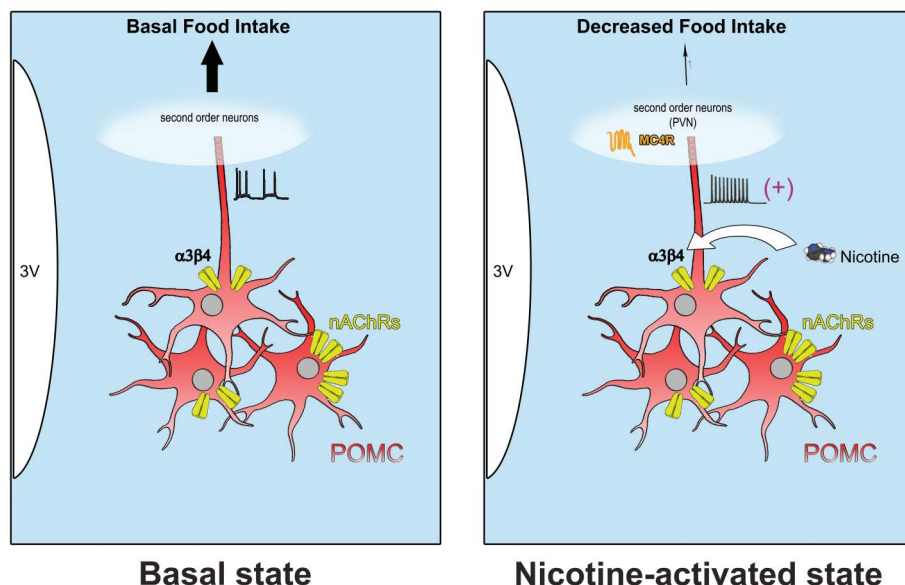


**Fig. 2.** Knockdown of  $\beta 4$  nAChRs in the ventral hypothalamus. We used AAV to deliver shRNAs to knock down the  $\beta 4$  subunit in the ventral hypothalamus. Sites of infusion were verified by GFP detection (photograph; 3V, third ventricle). After knockdown of  $\beta 4$  nAChRs and recovery, cytosine (1.5 mg/kg) was unable to decrease food intake, contrary to what was seen in the control group of animals. \*\*\* $P < 0.001$ . Error bars indicate SEM.



**Fig. 3.** POMC neuron activation by nicotinic drugs. Administration of nicotine (0.1 mg/kg) or cytosine (1.5 mg/kg), unlike mecamylamine (1 mg/kg), resulted in activation of POMC cells in the ARC as measured by c-fos immunoreactivity (A). Electrophysiological studies further demonstrate a dose-dependent [see (B) inset], reversible increase in the firing rate of identified POMC neurons in response to nicotine (B) or cytosine (C) application. \* $P < 0.05$ ; \*\* $P < 0.01$ . Food intake was not significantly affected by nicotine or cytosine in POMC KO

mice at two different doses (D). Furthermore, knockdown of Mc4r by AAV-shRNAs in the PVN [detected by GFP fluorescence (fig. S7)] significantly blunted the hypophagic response to nicotine over time compared with the response of mice injected with control virus and treated with nicotine. No signs of tolerance to nicotine were observed over 30 days, consistent with a role for MC4R signaling in the anorexic effects of nicotinic agents (E). \*\*\* $P < 0.001$ . Error bars indicate SEM.



**Fig. 4.** Hypothetical model of the anorexogenic effect of nicotine in the ARC. POMC neurons express nAChRs and therefore respond to nicotinic drugs. In the basal state (i.e., in the absence of nicotine), POMC neurons project to second order neurons that decrease food intake. When nicotine reaches the ARC (facilitated by its proximity to the third ventricle), activity of POMC neurons is increased (as measured by *c-fos* expression and neuronal activity measured in slices) through activation of  $\alpha 3\beta 4$  nAChRs and subsequent activation of MC4 receptors in the PVN of the hypothalamus (nicotine-activated state).

had a significant effect on the frequency and amplitude of mEPSCs (fig. S6, B and C, respectively).

To determine whether POMC neurons and melanocortin pathways were necessary for nicotinic-induced hypophagia, we treated POMC knockout (KO) mice with different doses of nicotine or cytosine and measured food intake over 24 hours (Fig. 3D). POMC KO mice showed no significant difference in food intake in response to nicotine [ $F_{2,16} = 0.56$ ,  $P = 0.58$ ] or cytosine [ $F_{2,20} = 0.78$ ,  $P = 0.46$ ] treatments, whereas cytosine-treated wild-type mice showed a decrease in food intake at each of the concentrations tested [at 1.5 mg/kg,  $F_{1,8} = 82.5$ ,  $P < 0.001$ ; at 3 mg/kg,  $F_{1,8} = 57.1$ ,  $P < 0.01$ ]. Lastly, we confirmed that release of melanocortin is critical for nicotinic-induced hypophagia by using AAV-shRNA delivery to knock down expression of the widely expressed melanocortin 4 receptor (Mc4r; fig. S7) in the paraventricular nucleus (PVN), where efferents of POMC neurons are present (16). Chronic treatment induced a blunting of nicotine-induced hypophagia in mice with Mc4r knockdown in the PVN [knockdown versus control:  $F_{10,180} = 5.54$ ,  $P < 0.0001$ ; Fig. 3E]; a similar pattern was observed in response to acute cytosine (1.5 mg/kg) [fig. S7;  $F_{1,18} = 10.05$ ,  $P = 0.005$ ].

Previous reports demonstrated that Mc4r activation by melanocortins is critical for the regulation of food intake and energy expenditure

(17), as confirmed by a trend for increased feeding at baseline after Mc4rKD in PVN. These data demonstrate that nicotinic drugs decrease food intake primarily through  $\beta 4^*$  (where \* represents other nAChR subunits still to be identified) nAChR-dependent activation of POMC neurons and melanocortin pathways. It has been demonstrated that POMC neurons express cholinergic markers (18) and that the naturally obese Tub/Tub strain of mice displays a decrease of perivascular cholinergic innervation in the ARC (19). These observations underscore a possible role for acetylcholine in metabolic regulation through POMC neurons (Fig. 4). It has also been suggested that cholinergic projections to the ventral hypothalamus could be provided by very localized groups of neurons found in the median eminence (20), a region harboring cells secreting hypophysiotropic hormones, including corticotropin-releasing hormone, all known to affect metabolism. Postsynaptic modulation of POMC neurons could also occur through cholinergic projections emanating from the pedunculopontine tegmental and laterodorsal tegmental nuclei (Ch5 and Ch6), regions that can adapt rapidly to metabolic stimulation (21, 22) and that are also involved in feeding behavior (23). All these mechanisms could therefore alter activity of POMC neurons and neurotransmitter release from presynaptic terminals that could, in turn, affect energy expenditure and feeding pat-

terns. Our results further suggest that  $\beta 4^*$  nAChRs are critical receptors mediating these effects.  $\alpha 3\beta 4$  agonists may therefore be useful for limiting weight gain after smoking cessation, and nicotinic drugs could also be useful to control obesity and related metabolic disorders.

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## Supporting Online Material

[www.sciencemag.org/cgi/content/full/332/6035/1330/DC1](http://www.sciencemag.org/cgi/content/full/332/6035/1330/DC1)  
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Figs. S1 to S7

References (24–35)

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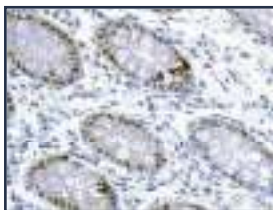
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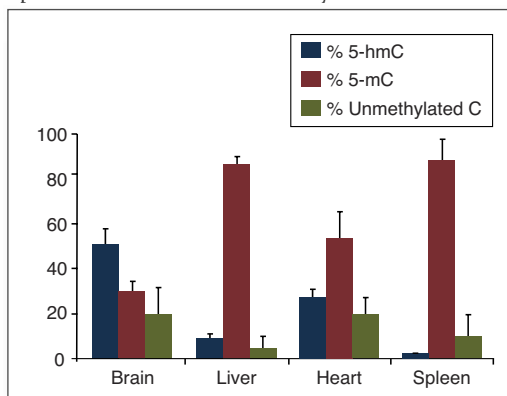


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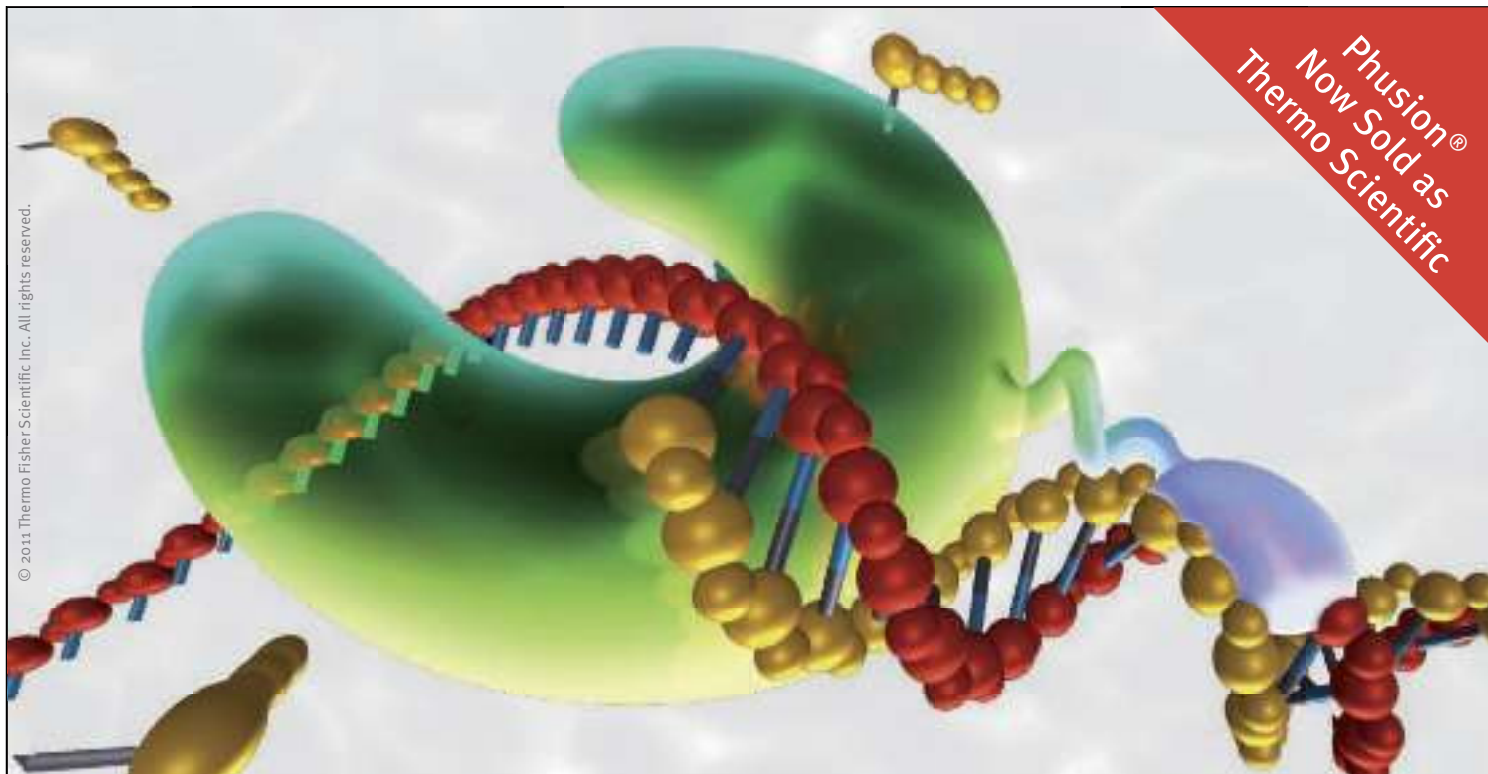
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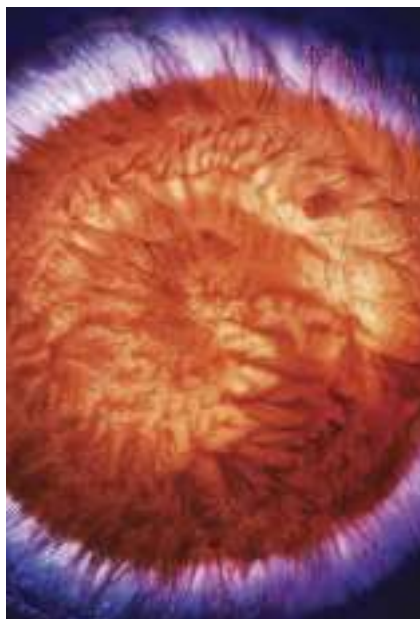
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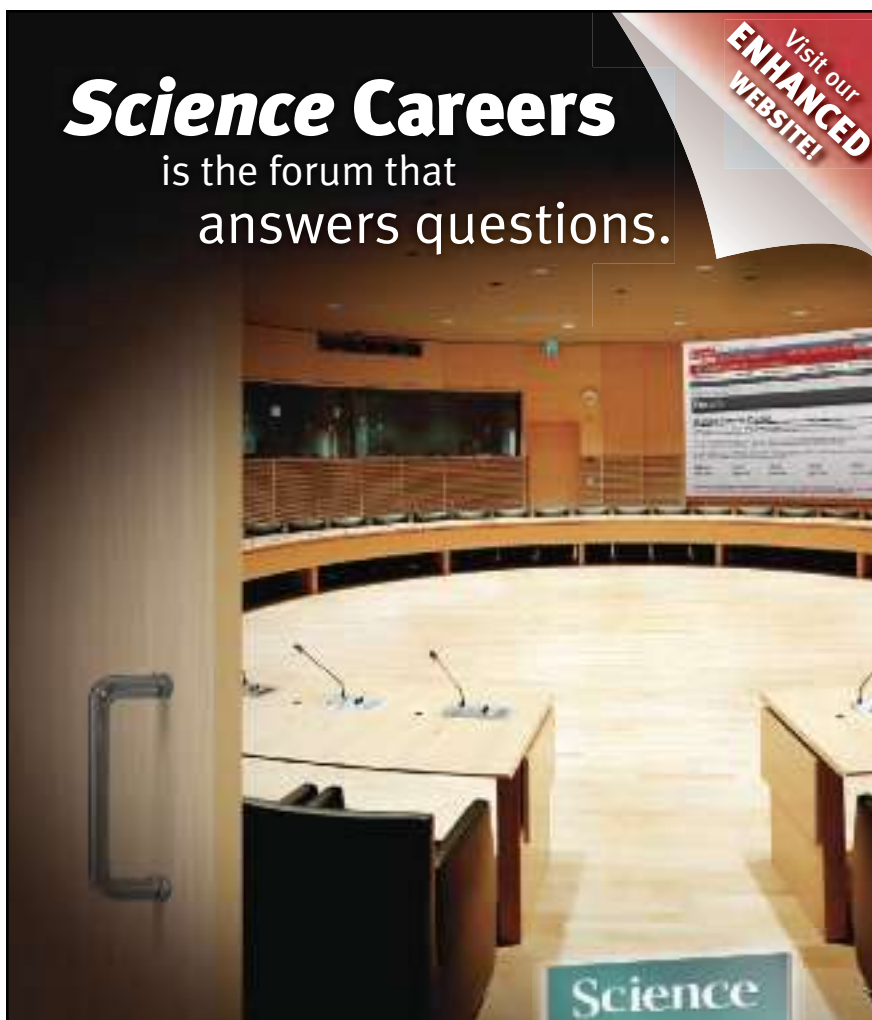
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**University Grants Commission** of India invites applications from outstanding highly motivated individuals, with demonstrated flair for research and teaching, for **positions of Assistant Professor, Associate Professor and Professor** in areas of basic sciences, i.e. Physics, Mathematics, Chemistry, Biology, and Engineering and Earth Sciences.

These positions have been created as part of a new Initiative designed to augment faculty resources in Indian Universities. The intent is to induct exceptional candidates with notable record of research in interdisciplinary/frontier areas of science, who will spearhead internationally competitive research programmes along with their teaching responsibilities.

Initial appointments at each level shall be for a period of 5 years, extendible through peer evaluation by successive 5-year terms. There is provision for elevation to next higher rank following end-of-term or mid-term appraisal of those occupying the position of Assistant/Associate Professor. The positions are tenable till the age of superannuation (presently at 65 years).

The University Grants Commission will synergize and coordinate with Institutions under its umbrella for appropriate placement of successful candidates.

The UGC-faculty will receive emoluments at par with those of the Central University appointees. In addition, facilities (such as residential accommodation, etc.), commensurate with those available to faculty of the host Institution, may be extended to them. Also, they will be entitled to competitive start-up grant for research.

For expanded description of the Programme, Positions and Application Procedure, please visit website: [www.ugcfrp.ac.in](http://www.ugcfrp.ac.in)

**Contact Address:** UGC-Faculty Recharge Programme  
Old CRS Building,  
Jawaharlal Nehru University,  
Aruna Asaf Ali Marg, New Delhi – 110 067, India  
E-mail: [encor@ugcfrp.ac.in](mailto:encor@ugcfrp.ac.in)



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**Georgia Health  
Sciences University**

**MEDICAL COLLEGE OF GEORGIA  
CANCER CENTER**

**Faculty Position in Proteomics and Metabolomics**

The Georgia Health Sciences University, Medical College of Georgia, Cancer Center is seeking applications for a position at the Assistant or Associate Professor level in the field of cancer Proteomics/ Metabolomics. Previous experience in this area is essential. Academic rank will be commensurate with experience. The successful candidate will join a team of experts in genomics and epigenomics to undertake a systems biology analysis of human cancer. It is anticipated that applicants would have a strong history of collaborative research, publications and NIH funding, ideally from NCI. A competitive start-up package is available to support this position.

A detailed description of the Georgia Health Sciences University, Medical College of Georgia, Cancer Center faculty and facilities is available at <http://www.georgiahealth.edu/cancer/>.

Inquiries should be addressed to **Dr. John K. Cowell**, Chair of the Search Committee, at [jcowell@georgiahealth.edu](mailto:jcowell@georgiahealth.edu) and should include detailed CV, names of three referees and a statement of research plans and career objectives. Additionally, all interested candidates should apply online at <http://www.georgiahealth.edu/facultyjobs/>, Requisition (reference) #5410.

**Application Deadline:** Until Filled

*Georgia Health Sciences University is an Equal Opportunity and Equal Access Institution; AA/EEO/Equal Access or AA/EEO/Equal Access/ADA Employer.*

**Stanford Stem Cell and Regenerative Medicine  
Institute/Stanford Cardiovascular Institute  
Cardiovascular Stem Cell Biologist  
with M.D., Ph.D., or M.D./Ph.D.  
Assistant Professor/Associate Professor**

The Stanford Stem Cell and Regenerative Medicine Institute and the Stanford Cardiovascular Institute seek to jointly recruit a cardiovascular stem cell biologist or cardiac physiologist with M.D., Ph.D., or M.D./Ph.D. degrees for this tenure-eligible position at an open rank. Applicants should have a strong record of research in molecular and cellular cardiovascular science and expertise or interest in using embryonic stem cells, with adult stem cells, embryonic stem cells, or/and inducible pluripotent stem cells in cardiovascular applications to study questions in cardiovascular biology. Other areas of expertise include electrophysiology, pharmacology, or cellular/molecular cardiology. Successful candidates will be expected to develop an independent research program using stem cells to address basic and translational issues in the cardiovascular domain medicine. This program will be an important collaborative component to the current cardiovascular regenerative medicine program. Candidate will join a dynamic group of scientists using stem cells to study a broad variety of questions and should focus on issues of directed differentiation and cell characterization.

Lab space for this new research program will be provided in the recently completed Lorry I. Lokey Stem Cell Research Building (SIM1). The SIM1 building consists of four floors adding up to a total of 200,000 gsf (130,000 nasf).

Interested applicants should send a cover letter and C.V. and the names of three references to: **Thomas Quertermous, M.D., Chief (Research), Division of Cardiovascular Medicine, C/O Corrine Sanchez, Department of Cardiothoracic Surgery, CVRB, Falk Bldg., Mail Code 5407, 300 Pasteur Drive, Stanford, CA 94305-5407.** Send by e-mail to: [corrine.sanchez@stanford.edu](mailto:corrine.sanchez@stanford.edu).

*Stanford University is an Equal Opportunity Employer and is committed to increasing the diversity of its faculty. It welcomes nominations of and applications from women and members of minority groups, as well as others who would bring additional dimensions to the University's research, teaching, and clinical missions.*

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**Our World-Class  
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Since its inception, The Methodist Hospital Research Institute has challenged the notion of "by-the-book" medical research. Led by Mauro Ferrari, Ph.D., President and CEO, the Research Institute is a 440,000-square-foot research enterprise for The Methodist Hospital System in Houston, TX, and is affiliated with the Weill Cornell Medical College in New York City. Methodist is transforming medicine with emerging techniques, and a staff that is developing real treatments and cures every day. Our laboratories are equipped with advanced technology and facilities that include a cyclotron, pre-clinical and clinical imaging, flow cytometry and microscopy, small and large animal vivariums; and a GMP facility for nanoparticles, contrast agents, vaccines, and therapeutic molecules. Our facility is a vertically integrated state-of-the-art laboratory for translational and clinical research where translational researchers and physician scientists bring ideas to clinical applications.

*We are now searching for research professionals (full professor equivalent) to serve in a variety of capacities.*

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in the fields of:**

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- **Cardiovascular Science (Methodist DeBakey Heart & Vascular Center)**
- **Cancer Biology (Methodist Cancer Center) — Filled by National Academy Members and CPRIT Scholars - Dr. Neal Copeland and Dr. Nancy Jenkins**
- **Diabetes and Metabolic Disorders (Methodist Diabetes & Metabolism Institute)**
- **Transplant Immunology (Methodist Transplant Center)**

Candidates should be nationally and internationally recognized leaders with an outstanding track record of scientific discovery, funded research, programmatic leadership and academic mentorship. We will provide you with a position in the epicenter of medical research. You'll discover an excellent research environment, state-of-the-art equipment, and the chance to follow your research from discovery to clinical application in a single facility.

Applicants should submit a Statement of Scientific Interest, a Curriculum Vitae, and the names of three references to: **Tong Sun, Director of Central Research Administration, The Methodist Hospital Research Institute, 6670 Bertner St., M.S. R2-216, Houston, TX 77030, or email [facultyapplications@tmhs.org](mailto:facultyapplications@tmhs.org) (please specify applying field in the subject line of email).** Our success as an organization is due to the diversity of our team. We are an equal opportunity employer.

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**DEPARTMENT OF HEALTH AND HUMAN SERVICES  
NATIONAL INSTITUTES OF HEALTH (NIH)  
National Center for Complementary and Alternative Medicine**



The National Center for Complementary and Alternative Medicine (NCCAM), a component of the National Institutes of Health (NIH), Department of Health and Human Services, seeks an accomplished, innovative neuroscientist to serve as Scientific Director of its Division of Intramural Research (DIR). The Scientific Director will build and lead a vibrant research program focused on the mechanisms and management of pain. This Scientific Director reports to the Director, NCCAM, and will also serve as a member of the NCCAM senior leadership team.

The Scientific Director will develop an overall vision for, and implement a research program focused on increasing understanding of the mechanisms of pain and its central modulation, with the long term goal of strengthening clinical management of chronic pain through the integration of pharmacological and non-pharmacological approaches. Topics of special interest include pathways and mechanisms by which emotion, attention, and other processes modulate pain or pain processing, and mechanisms of the placebo effect. The research program will be highly collaborative with, and leverage the basic and clinical research talent and resources of other ongoing neuroscience and imaging programs of the larger NIH intramural research community.

This exceptional opportunity is available to an accomplished neuroscientist who has a demonstrated track record of internationally recognized research on pain, a commitment to both basic and clinical research, and the leadership and management skills to build and sustain vibrant, collaborative team efforts with colleagues within intramural programs across the NIH.

Applicants must possess an M.D., Ph.D., or equivalent degree in the biomedical sciences, and have professional experience reflecting a broad scientific background and experience in basic and/or clinical neuroscience research. Applicants should be known and respected within their profession, both nationally and internationally, as distinguished individuals of outstanding scientific accomplishment. Salary is commensurate with experience and a full package of Civil Service benefits is available including retirement, health and life insurance, long term care insurance, leave and savings plan (401 K equivalent).

**Application Process:** Interested candidates should send a letter of interest, including a brief description of research and administrative experience, CV, bibliography, and a list of up to five individuals who can serve as references to: **Ms. Belinda Davis** at [nccamsrrecruits@mail.nih.gov](mailto:nccamsrrecruits@mail.nih.gov). Email receipt of applications and inquiries is preferred; however, candidates needing reasonable accommodation may fax application materials to **301-402-4741**.

Applications will be reviewed starting **June 27, 2011**, and will be accepted until the position is filled. All information provided by applicants will remain confidential and will not be released outside the NCCAM search process without a signed release from the applicant.

*The NIH encourages the application and nomination of qualified women, minorities, and individuals with disabilities.  
NIH AND DHHS ARE EQUAL OPPORTUNITY EMPLOYERS*



United Nations  
Educational, Scientific and  
Cultural Organization

## **REGIONAL CENTRE FOR BIOTECHNOLOGY**

**an institution of education, training & research**

(Established by the Dept. of Biotechnology, Govt. of India under the auspices of UNESCO)

### **Career Opportunities in Data-Intensive Discovery Research**

The Regional Centre for Biotechnology (RCB), a newly created institution in the National Capital Region (NCR) of Delhi, is looking for innovative faculty members working in the domains of data-intensive scientific discovery to develop novel research and teaching programmes in the area of biotech science. The RCB recognizes that the expertise and innovation in the core domains of systems biology, computational biology, bioinformatics and other branches of theoretical biology and analyses of clinical/epidemiological/biodiversity data is critical for the development of novel perspectives in biotechnology, and invites scientists with interdisciplinary profiles of the highest caliber and credibility to participate in this shared adventure to transform the biotech sciences. A description of the RCB and its mandates and projections can be found on the website: <http://www.rcb.res.in>

Faculty appointments will be offered from entry to program coordinator levels depending on the quantum of experience and the quality of scientific productivity. Interested scientists are invited to send their curriculum vitae containing relevant details, list of publications and the names of three potential referees along with their electronic mail addresses to the **Executive Director, Regional Centre for Biotechnology, 180, Udyog Vihar, Phase-I, Gurgaon- 122016, India**. An advance electronic copy of the bio-data may be sent to the e-mail address: [office@rcb.res.in](mailto:office@rcb.res.in). Only short-listed candidates will be contacted for further discussions.

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## SYMPOSIUMS

### **NSF PIRE Summer School Program in Cognitive, Computational and Systems Neuroscience (PICCS) September 3-10, 2011**

Join us for a week of seminars and scientific discussion with some of the world's leading neuroscientists at a summer retreat in Bavaria, Germany. Thirty lectures and seminars over six days with ample recreation time and cultural excursions provide the ideal background for learning and networking. The Summer School is hosted at a monastery on the lake island Frauenchiemsee in the foothills of the Bavarian Alps, providing an intimate environment for conversation and collaboration.

**Topics:** Sensory Systems, Cortical Plasticity, Computational Neuroscience, Functional Neuroimaging.

**Keynote Speakers:** Rodolfo Llinás (NYU), Nikos Logothetis (MPI Tübingen), Bill Newsome (Stanford U.), Dietmar Plenz (NIH), Robert Zatorre (McGill U.).

**Organizing Committee:** Josef Rauschecker (Georgetown U.), Werner Graf (Howard U.), Rhonda Dzakpasu (Georgetown U.).

Apply at: [pire-georgetown-howard.org](http://pire-georgetown-howard.org)





## PROGRAM LEADER International Institute for Applied Systems Analysis

The International Institute for Applied Systems Analysis (IIASA, [www.iiasa.ac.at](http://www.iiasa.ac.at)), located near Vienna, Austria, is seeking a Program Leader for its newly created Water (WAT) Program beginning autumn 2011. The successful candidate will work closely with the Food and Water Area Leader and other Program Leaders to build a team of researchers with a focus on global water security, integrated management of water resources in arid and semi-arid areas, and management of water resources used by multiple jurisdictions and nations. In addition to developing models, databases and analysis capabilities to support IIASA research in the major global problem areas of energy and climate change, food and water, and poverty and equity, the Program Leader will coordinate the allocation of the internal budget for the Water Program and attract external research funding to supplement and expand the core budget and develop international and in-house cooperation and networks on integrated water resource management as it relates to IIASA's research topics and programs.

Candidates should combine a vision for IIASA's Water Program with scientific excellence, management and diplomatic skills, and broad experience in interdisciplinary research and policy applications in the international arena.

IIASA is nongovernmental, sponsored by an international consortium of 18 National Member Organizations. Applicants should have excellent written and spoken English, IIASA's working language. The Institute's management and staff alike are committed to a working environment that promotes equality, diversity, and tolerance.

An initial contract of 3 years will be offered with the possibility of extension. IIASA offers a competitive compensation and benefits package and salaries are exempt from taxation in Austria. Review of applications will begin immediately. To apply, email a cover letter, CV, contact details of 3 work-related reference givers and copies of 5 key publications to:

**Ms. Alia Harrison, Recruitment Officer**  
International Institute for Applied Systems Analysis (IIASA)  
Email: [harrison@iiasa.ac.at](mailto:harrison@iiasa.ac.at)

## MEDICAL SCHOOL FACULTY

### St. Kitts Medical Campus

The University of Medicine and Health Sciences is a premier, for-profit boutique off shore medical school located on the exotic Caribbean Island of St. Kitts. Our ocean front state of the art campus is comparable to the best medical schools in the United States. We maintain small classes and provide a personalized education catering to the individual needs of the students. To date we have invested over 50 million dollars into our campus facilities and infrastructure. we are seeking the following full time faculty:

### MEDICAL MICROBIOLOGY BIOCHEMISTRY PHYSIOLOGY

Preference will be given to candidates who have a minimum of 4 years experience teaching in a U.S. or Canadian medical school. Candidates must have a minimum of an earned PhD in their discipline. Candidates additionally holding an M.D. degree will be given preference. Salaries and benefits are competitive and commensurate with experience. A significant portion of the salary is offered tax free.

Candidates should forward their CV's  
and cover letter to: [hr@umhs-sk.net](mailto:hr@umhs-sk.net)

For further details about

### The University of Medicine and Health Sciences

For further details about our university please visit [www.umhs-sk.org](http://www.umhs-sk.org)

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## CONFERENCE



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### Event at a Glance

#### Program Overview

Exhibition (COEX 3F Hall C) : 300 Companies 550 Booths

Conference (COEX 3F Conference Room) : 15 Tracks 50 Sessions

Business Forum (COEX 3F Hall E) : Presentation and Partnering (Matching)

#### Organized by

Korea International Trade Association (KITA)

Korea Health Industry Development Institute (KHIDI)

Chungcheongbuk-do (Chungbuk)

#### Supported by

Ministry of Knowledge Economy (MKE)

Ministry of Health & Welfare (MHW)

Seoul Metropolitan Government

### Point of Contact

**Exhibition** Tel. 82-2-6000-5275 e-mail : [biokorea@kita.net](mailto:biokorea@kita.net)

**Conference** Tel. 82-2-508-4217 e-mail : [bioconf@ibimp.com](mailto:bioconf@ibimp.com)

**Biz Forum** Tel. 82-2-508-4217 e-mail : [biobiz@ibimp.com](mailto:biobiz@ibimp.com)





## Nontraditional Careers: Opportunities Away From the Bench Webinar

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### POSITIONS OPEN

#### DIRECTOR

#### Warfighter Protection & Applications Division (Pharmacologist, Physiologist, or Biologist)

The Office of Naval Research is seeking a qualified individual to serve as the Director of the Warfighter Protection & Applications Division in the Warfighter Performance Science & Technology (S&T) Department. The incumbent is responsible for managing and directing extensive activities in fostering, administering, and executing an integrated program of basic research, applied research, and advanced technology development in the fields of biology, physiology, pharmacology, and research psychology.

Sponsored efforts are conducted principally at U.S. universities and industry or Federal laboratories. This is a Civil Service position at the NP-IV level (\$105,211–\$155,500) depending on individual qualifications. The position requires a world-class scientist with knowledge and experience in the fundamental theories, concepts, and current-state-of-the-art research and/or technology development in the broad areas of biology, physiology, pharmacology, and research psychology, and has a proven track record in carrying out research and development at a level that has garnered respect in the highest levels of academia.

For information on qualifications and how to apply, see the Job Announcement via the link posted at website: <http://www.onr.navy.mil/en/career-job-opportunity/job-listing.aspx>. U.S. citizenship required. An Equal Opportunity Employer.

#### RESEARCH SCIENTIST

#### Hitachi Chemical Research Center, Inc.

Hitachi Chemical Research Center, Inc. (HCR) is a research and development company directed towards novel technology platforms and related bio or bio mimetic materials for life sciences. HCR is a subsidiary of Hitachi Chemical Company, Ltd., a chemical manufacturer producing innovative technologies in the areas of Electronics Related, Automotive Related, and Advanced Performance Products.

HCR is hiring a Research Scientist with a degree in biochemistry with a strong background in polymer science and polymer synthesis. The researcher will be joining a research team who is developing polymer based sensors and OPV materials. We are looking for a candidate with the following qualifications: A Ph.D.; demonstrated scientific creativity and technical proficiency in his/her field as evidenced by publications in top tiered journals and presentations of work at relevant scientific conferences; advanced problem solving skills, excellent communication skills, and sound scientific judgment of complex issues; background working on a multidisciplinary research project.

HCR, located in Irvine, California offers competitive benefits and salary. Interested candidates can electronically send resumes to Ms. Lisa Osborn at e-mail: [recruiting@hrcrcenter.com](mailto:recruiting@hrcrcenter.com) or telephone: 949-725-2727. Equal Opportunity Employer.

**RESEARCH ASSOCIATE POSITION.** We are looking for an energetic and highly motivated scientist to participate in the chemical genomics drug discovery project. The candidate must have working experience in drug research, preferably related to either GPCRs or stem cells. A background in either computational biology, medicinal chemistry, or protein biophysics using LC/MS or others is required. Excellent communication and writing in English are required. Salary will be commensurate with experience. Please electronically send your curriculum vitae and three references to: Professor XQ (Sean) Xie, Department of Pharmaceutical Sciences/Drug Discovery Institute, University of Pittsburgh, Pittsburgh, PA 15261 at e-mail: [xix15@pitt.edu](mailto:xix15@pitt.edu). Websites: <http://www.cbligand.org/xielab/> and <http://www.pharmacy.pitt.edu/directory/profile.lasso?Page=425&Type=Faculty>. The University of Pittsburgh is an Affirmative Action/Equal Opportunity Employer.

### POSITIONS OPEN

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